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Lymphocytic foci in the endometrium of pregnant dairy cows: Characterization and association with reduced placental weight and embryonic loss

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ABSTRACT

Lymphocytic foci (also known as lymphoid aggregates or tertiary lymphoid structures) form within the bovine endometrium after antigenic challenge. Their presence in the pregnant uterus provides evidence for a chronic inflammatory condition perhaps arising from an early postpartum uterine infection. The chronic inflammation that includes the foci could explain greater embryonic loss in dairy cows with early postpartum uterine disease. The objectives were to characterize the size and location of the foci in the pregnant uterus, determine their composition using immunohistochemistry, and associate their presence with the development of the pregnancy and embryonic loss. Pregnant cows (n = 43) were slaughtered on days 28, 35, or 42 of pregnancy. Uterine tissue was collected and processed for histologic and immunohistochemical analysis. The number of small (<100 micron diameter), intermediate (100–250 micron diameter), and large (>250 micron diameter) foci was counted. The number of cows averaging 0, 0.1 to 1, 1.1 to 2, and more than 2 foci per section (small, intermediate, and large; combined) was 7 (16%), 14 (33%), 11 (26%), and 11 (26%), respectively. The average number of small and intermediate foci found in the histologic sections was greater in cows with evidence of uterine infection postpartum ($P < 0.05$). Lymphocytic foci were distributed within the caruncle and the intercaruncular tissue and comprised a core of CD3-positive cells (T cells) surrounding CD79-positive cells (B cells). The number of lymphocytic foci was correlated with a total inflammation score (on the basis of the total number of inflammatory cells in the endometrium; $r^2 = 0.49$; $P < 0.001$) and a fibrosis score (based on the extent of fibrosis in the endometrium; $r^2 = 0.33$; $P < 0.001$). Cows with a high foci count (averaging more than 0.5 foci per section) had lesser ($P < 0.01$) placental weight on Day 42 of pregnancy. There was no effect of foci count on placental weight on Day 28 or 35. Two cows with embryonic loss were in the highest quartile for foci count. In conclusion, cows with chronic inflammation as evidenced by a large number of lymphocytic foci had reduced placental weight during pregnancy. The number of foci in pregnant cows was associated with early postpartum uterine disease. Whether the foci themselves are inhibitory to pregnancy development or are associated with other bacteriological, morphological, or biochemical changes to the uterus that lead to infertility will need to be investigated.

1. Introduction

Nearly all cows have pathogenic bacteria in the uterus postpartum [1–3]. In response to the pathogenic challenge, one-third to one-half of cows develop some manifestation

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of uterine disease including metritis, purulent vaginal discharge (PVD), and (or) endometritis [4]. The remaining cows remain healthy (no overt uterine disease). Cows that develop uterine disease suffer from long-term infertility [5,6]. One mechanism that leads to infertility in previously diseased cows is early embryonic loss where cows are diagnosed pregnant with an ultrasound examination at 28 to 35 days and are later found not pregnant. Both Ribeiro et al. [7] and Machado et al. [8], for example, found that cows with vaginal discharge at 30 days postpartum had more pregnancy loss between 30 and 35 days (first diagnosis) and 65 days (second diagnosis) after AI compared with healthy control. The long-term infertility associated with uterine disease postpartum may involve a mechanism through which the previous infection can create a functional change within the uterus that antagonizes the establishment of pregnancy and (or) causes early embryonic loss.

In a previously published study, we tested the effects of lactation on the development of the conceptus on Day 28, 35, or 42 of gestation in postpartum dairy cows [9]. In that study, postpartum dairy cows were sacrificed on different days of gestation and tissues were collected. Most of the tissues were subjected to analyses of gene expression [10]. In addition, samples were fixed for histology. We found lymphocytic foci (clusters of immune cells also referred to as lymphoid aggregates [11] or tertiary lymphoid structures [12]) within the endometrium of pregnant cows. Lymphocytic foci within the bovine endometrium were described previously by Bonnett et al. [13]. In their study of the postpartum bovine uterus on days 26 and 40, they reported a positive association between the foci and the presence of anaerobic bacteria in the uterine lumen on Day 40 postpartum [13]. In later work, Walker et al. [14] reported that cows with endometritis had an average of 3.67 ± 1.04 foci in the stratum spongiosum compared with 0.28 ± 1.04 in healthy control ($P < 0.04$). Lymphocytic foci are known to exist within the human uterus as well, where they form in response to infection and may persist for months or years after infection [11]. They comprised a B-cell core surrounded by memory T cells [11]. In studies where heifers were challenged with *Tritrichomonas foetus*, lymphocytic foci formed and persisted for at least 10 weeks after infection [15]. The authors concluded that the foci formed after the antigenic challenge and required sustained antigen stimulation to develop. Earlier work from the same laboratory concluded that lymphocytic foci were inductive sites for immune response [16]. The lymphocytic foci are similar to other inductive sites, therefore, including gut-associated lymphoid tissues (Peyer's patches) that initiate mucosal T- and B-cell responses [17].

Lymphocytic foci in the pregnant uterus may represent cytologic evidence for postpartum uterine infection that occurred early postpartum. To better understand the foci themselves and potential implications for the development of the pregnancy, we initiated an investigation into the lymphocytic foci during pregnancy in the dairy cow. First, we characterized the size and location of the foci in the pregnant uterus; second, we determined their composition using immunohistochemistry; and finally, we associated their presence with uterine tissue morphology, development of the pregnancy, and embryonic loss.

2. Materials and methods

2.1. Animals and treatments

The project was approved by the University of Missouri–Columbia Animal Care and Use Committee. A detailed description of the experiment has been reported [9]. Pregnant Holstein heifers ($n = 43$) were randomly assigned to one of two treatment groups, either lactating ($n = 23$) or not lactating (nonlactating; $n = 20$). The nonlactating group was not milked after calving (i.e., dried off immediately, never milked). Both groups were housed in the same pen and fed the same diet that was formulated for lactating cows. The reproductive tract of each cow was examined weekly using transrectal ultrasonography (Aloka 500-SSD equipped with 7.5 MHz transducer; Aloka, Tokyo, Japan). If PVD, fever, and (or) foul odor coming from the uterus early postpartum was present, then it was recorded at the time of the ultrasound examination. All cows were inseminated from a single ejaculate of a high fertility sire following a timed AI program. Cows that did not conceive to first insemination were subsequently re-inseminated 12 hours after an observed return to estrus or after a timed AI. Pregnant cows were slaughtered at one of 3 days of pregnancy (28 days [lactating, $n = 7$; nonlactating, $n = 7$], 35 days [lactating, $n = 8$; nonlactating, $n = 6$], or 42 days [lactating, $n = 8$; nonlactating, $n = 7$]).

Cows were taken to the University of Missouri abattoir in the morning where they were stunned by using a pneumatic captive bolt and then killed by exsanguination. The reproductive tracts were collected, placed on crushed ice, and taken to the laboratory. The pregnancy (placental membranes encasing placental fluids and fetuses) was removed from the uterus. The pregnancy was considered viable if the embryo had a normal appearance (well vascularized with clear fluid). There were two cows with apparent embryonic loss (dead embryo) as evidenced by brown necrotic embryonic and placental tissue in the uterine horn. The fetus and placenta from viable pregnancies were weighed. Samples of endometrium that included both caruncular and intercaruncular areas were fixed in 10% neutral buffered formalin. The samples were collected from the pregnant horn ($n = 3$) and the nonpregnant horn ($n = 3$). Sites for collection were distributed throughout the horn.

The fixed tissues were trimmed to approximately 2 cm of endometrial surface that included a caruncle and intercaruncular region. The trimmed tissue was placed into a plastic tissue cassette and taken to the Veterinary Medicine Diagnostic Laboratory at the University of Missouri for routine processing and hematoxylin–eosin staining. Histologic sections from the pregnant horn ($n = 3$) and the nonpregnant horn ($n = 3$) were examined using a Leica DM 4000B microscope (Buffalo Grove, IL) fit with a Leica DFC 450C camera. Each slide was viewed at $\times$ 50 to count the number of foci and at $\times$ 200 magnification to measure the foci for the diameter classification. The number of lymphocytic foci within each cross section within specific diameter classifications was counted. The specific size of the lymphocytic foci was determined by comparing its size with a micrometer on the video image generated by the