EFFECT OF INDUCED PYOMETRA ON LUTEAL LIFESPAN AND UTERINE FLUID CONCENTRATIONS OF PROSTAGLANDINS AND INTERFERONS IN COWS

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ABSTRACT

The objective was to determine whether occurrence of persistent corpora lutea in cows with pyometra could be explained by accumulation of prostaglandins and interferons in the uterus. Pyometra was induced by treatment with hCG, followed by intrauterine infusions of iodine and Actinomyces pyogenes. Five of the seven treated cows developed pyometra as indicated by extended estrous cycles (30-37+ d) and presence of purulent uterine exudate at slaughter (Day 37 after estrus). Mean (± standard error) concentrations of prostaglandins E2 and F2α for the cows with pyometra were 34.7 ± 14.4 and 96.3 ± 28 ng/ml, while the concentrations in uterine flushings in the remaining 2 cows averaged 1.85 ± 0.7 and 0.9 ± 0.2 ng/ml, respectively. Based on Western blotting, none of the cows had detectable concentrations of either interferon-α or interferon-τ in uterine secretions. In conclusion, pyometra results in increased uterine prostaglandin secretion. While increased secretion of cytokines associated with bacterial infection may be contribute to this effect, there was no evidence in this study to suggest that interferons are involved in the mechanism which causes persistence of the corpus luteum in bovine pyometra.

Key words: prostaglandins, bovine, pyometra, interferon, uterus

INTRODUCTION

Pyometra is characterized by progressive collection of a purulent exudate in the uterine cavity, infection, and persistence of a functional corpus luteum (CL). Acyclicity, inflammation, and infertility are consequences of pyometra which can be a sequelae to postpartum endometritis.

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Bovine pyometra can be mistaken for pregnancy because both conditions are characterized by a period of anestrus and prolonged luteal lifespan. Reasons for retention of a functional CL are not well understood. Maintenance of the CL during pregnancy is caused by the actions of a conceptus interferon (interferon-τ) that inhibits production and release of uterine prostaglandin (PG) F₂α (8, 17, 34). Effects of interferon-τ (IFNτ) on CL lifespan (19) can be mimicked by intrauterine infusion of the related interferon, interferon-α (IFNα; 29, 30, 31). Thus, it is possible that extended CL lifespan in cattle with pyometra occurs because leukocytes infiltrating the infected uterus produce IFNα (27). Another possible factor in maintenance of the CL is prostaglandin E₂ (PGE₂). Elevated concentrations of PGE₂ have been found in uterine fluids of cows with pyometra (18, 24), and this hormone can extend luteal lifespan when infused into the uterus (16, 32, 38). Therefore, enhanced uterine synthesis of PGE₂ could lead to maintenance of the CL.

The objective of this experiment was to evaluate whether bovine pyometra is associated with increased uterine lumenal concentrations of IFNα, IFNτ and prostaglandins. The approach taken was to experimentally induce pyometra through infusion of A. pyogenes using a slightly-modified version of a previously published protocol (10).

MATERIALS AND METHODS

Growth of Bacteria

A lyophilized pellet of Actinomyces pyogenes (ATCC 25286; Hardy Diagnostics, Santa Marta, CA) was dissolved in 0.5 ml sterile trypticase soy broth (Difco, Detroit, MI). Purity was checked by swabbing a 5% sheep blood MacConkey agar plate. Inoculated broth was poured into 1 l sterile trypticase soy broth and incubated overnight at 37°C. Approximately 24 h later, the broth was centrifuged at 1000 x g for 15 min and the pellet was washed in sterile 10 mM KPO₄, 0.9% NaCl, pH 7.4 (PBS) and then PBS with 15% glycerol. Stocks were divided into aliquots and stored at -70°C until use. The stock solution was thawed and the concentration was evaluated by determining bacterial growth of serially-diluted samples of stock (growth of bacteria after the original stock was diluted by 10⁶ was considered indicative that the bacterial concentration of the stock was 10⁶ colony forming units/ml). The stock solution was then diluted to deliver 2 x 10⁸ CFU in 60 ml sterile saline.

Induction of Pyometra

A group of 12 nonlactating, multiparous Holstein cows possessing CL (detected by palpation per rectum) were injected with 25 mg, i.m. PGF₂α, (Upjohn, Kalamazoo, MI). Of these, 7 cows that exhibited estrus 48 to 96 h later were used for the experiment. On Day 12 postestrus (Day 0=onset of estrus), rectal palpations were performed to verify the presence of a CL, and cows received 10,000 IU i.m. hCG (Sigma Chemical Co., St. Louis, MO). On Day 14 after estrus, 255 ml of a 3.0% (w/v) iodine solution were infused into the uterus. An infusion pipette was inserted through the cervix for delivery of approximately 150 ml to the uterine horn ipsilateral to the CL and approximately 105 ml to the contralateral horn. On Days 15, 16 and 17, the animals received an intrauterine inoculation of A. pyogenes (2.0 x 10⁸ CFU/ml), in a total volume of 60 ml saline, by passing an infusion pipette into the uterus of each animal. The procedure for
inducing pyometra is very similar to one used earlier (10) except that the hCG was given on Day 12 instead of Day 10.

Detection of Estrus and Serum Collection

Twice-daily observations of estrus were made throughout the experimental period. Tailheads of the animals were marked with paint stick as an aid for detection of estrus. Once a week, the animals were subjected to ovarian palpation for presence of a CL. In addition, blood samples were collected twice weekly from the coccygeal vein for progesterone analysis. Blood samples were stored at 4°C for 1.5 to 2 h to permit clotting. The samples were centrifuged for 15 min at 400 x g. Serum was collected and frozen at -20°C for later analysis. Estrous cycle length was defined as the period from estrus prior to bacterial infusion until the next observed estrus or the first time serum progesterone concentration fell below 1 ng/ml.

Collection of Uterine Fluid

On Day 37, reproductive tracts were obtained from cows after exsanguination. Uterine fluid was removed from the tract by aspiration with a 14-ga needle and a 60-cc syringe. In 2 cows without visible uterine fluid, the uteri were flushed with approximately 40 to 45 ml 0.9% sterile saline. Uterine fluids and flushes were centrifuged at 10,000 x g for 30 min, and the supernatant was collected and frozen at -20°C until analysis.

Radioimmunoassays

Concentrations of progesterone in unextracted serum were determined using a commercial progesterone solid-phase radioimmunoassay (Diagnostics Products Corp., Los Angeles, CA) previously validated for use with bovine plasma (36).

For prostaglandin assays, aliquots of uterine fluid or flushings (250 μl for PGE₂, 500 μl for PGF₂α) were extracted through the addition of 2 ml distilled ethyl acetate and 20 μl 1.0 N HCl. Then, 8000 dpm [³H]PGE₂ (Amersham, Arlington Heights, IL; specific activity 184 Ci/mmol) or 10,000 dpm [³H]PGF₂α (Amersham; specific activity 208 Ci/mmol) were added to each tube to estimate extraction efficiency. Extraction and recovery tubes were vortexed for 4 min and centrifuged at 1000 x g for 10 min. The solvent phase was dried under N₂. Samples were then reconstituted with the same volume of 0.05 M Tris-HCl, pH 7.4, that was extracted and vortexed for 10 min. Extraction recoveries were 73 to 83% for PGE₂ and 31 to 73% for PGF₂α.

Diluted samples were analyzed for PGE₂ and PGF₂α by RIA according to procedures previously described (20). The assays utilized antibodies characterized previously for PGE₂ (23) and PGF₂α (15). Antiserum for PGF₂α crossreacted with PGF₁α by approximately 63% (15). Intra- and inter-assay coefficients of variation (2 assays for each prostaglandin) were 13 and 7% for PGE₂ and 15 and 12% for PGF₂α. The limits of detectable prostaglandin were 4 pg for PGE₂ and 2 pg for PGF₂α. Assays were validated by verifying parallelism of the standard curve with various dilutions of pooled samples. When known amounts of prostaglandins were added to the pooled sample, recoveries were 109 ± 16.3% for PGE₂ and 110 ± 10.0% for PGF₂α.
Western Blotting

Samples analyzed for presence of IFNα and IFNγ by Western blotting were as follows: uterine flushings and fluids of experimental animals (i.e., those receiving A. pyogenes); pooled samples of uterine flushings collected from cows at Day 17 of pregnancy (positive control) and Day 17 of the estrous cycle (negative control); 0.5 and 2.0 μg/lane rbIFNα or rbIFNγ (positive control). Recombinant bovine interferon-γ (rbIFNγ) was donated by R. M. Roberts (University of Missouri, Columbia, MO), while recombinant bovine interferon-α 1 (rbIFNα) was donated by CIBA-GEIGY (Basle, Switzerland). To verify that the pyometra fluids would not interfere with the detection of interferons, additional blots were run with pooled samples of uterine fluids in which 2 μg/lane rbIFNα or rbIFNγ were added. The samples were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by mixing 1:1 with 0.125 M Tris-HCl, pH 6.8 containing 20% (w/v) sucrose, 10% (w/v) sodium dodecyl sulfate, and 5% (v/v) p-mercaptoethanol and boiling for 3 min. Electrophoresis (20 μl diluted sample/lane) was performed using the buffer system of Laemmli (1970) (22) and 15% (w/v) polyacrylamide gels. After electrophoresis, proteins were transferred electrophoretically to Immobillon HP membranes (Millipore, Bedford, MA) at 200 mA overnight in buffer of 25 mM Tris, with 1.5% (w/v) glycine and 20% (v/v) methanol. Membranes were blocked with blotting buffer (20 mM Tris-HCl, pH 7.5 with 0.5 M NaCl, 0.05% [v/v] Tween-20) containing 1% (w/v) nonfat dry milk at 4°C overnight. Membranes were washed twice with blotting buffer and incubated overnight at 4°C with antisera or normal rabbit serum (diluted 1:1000 in blotting buffer containing 0.5% [w/v] bovine serum albumin). Antisera used were a rabbit polyclonal antibody to rbIFNα donated by CIBA-GEIGY (Basle, Switzerland) and rabbit polyclonal antibody to rbIFNγ produced in the laboratory. Membranes were washed again and incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma, St. Louis, MO; diluted 1:1000 in blotting buffer containing 0.5% [w/v] bovine serum albumin). Membranes were washed briefly with deionized water and incubated for 10 min with developer (3 mg/ml fast red and 0.05% [w/v] naphthol phosphate in 0.1 M Tris-HCl, pH 9.5 with 1.0 mM MgCl₂; Bio-Rad, Richmond, CA). Color development was stopped by rinsing blots in deionized water.

RESULTS

Estrus Cycle Length

Individual profiles of estrous behavior and serum progesterone concentrations are shown in Figure 1. None of the cows were observed for standing estrus during the trial. However, estrus was detected in 2 cows by removal of tailhead paint markings on Day 28 (Cow 8918) and Day 34 (Cow 5282). Two other cows experienced functional luteolysis (progesterone concentration below 1 ng/ml) at Day 30 (Cow 5082) and Day 37 (Cow 5117). Three animals (Cows 5225, 1726 and 1641) had a functional CL at end of the trial (Table 1). Thus, all the cows experienced extended estrous cycles.

Characteristics of the Reproductive Tract at Day 37

Two animals (Cows 8918 and 5282) were classified as not having pyometra—no fluid or pus had accumulated in the uterus at slaughter, and both cows had returned to estrus before slaughter (estrous cycle lengths of 28 and 34 d, respectively). Pyometra was induced in the remaining cows as indicated by the presence of a purulent exudate in the uterus and extended estrous cycles.
cycles (Table 1). Three of these uteri (from Cows 5082, 1641 and 1726) had fluid-filled abscesses extending from the endometrial wall towards the myometrium. In one of these reproductive tracts (Cow 1726), there were adhesions of the ovary and tip of the uterine horn to the uterine body and cervix. One animal (Cow 5082) possessed discrete liver abscesses at slaughter, but it is not known whether these were associated with the treatment to induce pyometra.
Table 1. Effects of induced pyometra on estrous cycle length, reproductive tract, uterine fluids and prostaglandin secretions.

<table>
<thead>
<tr>
<th>Cow ID no.</th>
<th>Pyometraa</th>
<th>Length of estrous cycle (days)b</th>
<th>Characteristics of the reproductive tractc</th>
<th>Uterine Fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Volume (ml)</td>
</tr>
<tr>
<td>5282</td>
<td>-</td>
<td>34</td>
<td>No CL or apparent infection</td>
<td>0d</td>
</tr>
<tr>
<td>8918</td>
<td>-</td>
<td>28</td>
<td>2-CL, no apparent infection</td>
<td>0d</td>
</tr>
<tr>
<td>5117</td>
<td>+</td>
<td>37</td>
<td>No CL; clear fluid in uterus</td>
<td>62</td>
</tr>
<tr>
<td>5082</td>
<td>+</td>
<td>30</td>
<td>CL; abscess and brown fluid in uterus</td>
<td>141</td>
</tr>
<tr>
<td>1641</td>
<td>+</td>
<td>&gt;37</td>
<td>CL; abscess and thick yellow fluid in uterus</td>
<td>12</td>
</tr>
<tr>
<td>5225</td>
<td>+</td>
<td>&gt;37</td>
<td>CL; thick brown fluid in uterus</td>
<td>544</td>
</tr>
<tr>
<td>1726</td>
<td>+</td>
<td>&gt;37</td>
<td>CL; abscess, adhesions and thick white fluid in uterus</td>
<td>184</td>
</tr>
</tbody>
</table>

a As determined by an extended estrous cycle and purulent material in the uterus.
b Interval from estrus until next estrus (Cows 8918 and 5282) or until serum progesterone concentration was less than 1 ng/ml.
c At slaughter (d 37 after onset of first estrus).
d Reproductive tract was flushed with approximately 40 to 45 ml of saline.

Prostaglandin Concentrations in Uterine Fluids or Flushes

Concentration of prostaglandins (± standard error) in the uterine fluids of cows with pyometra (n=5) averaged 34.7 ± 14.4 for PGE₂ and 96.3 ± 28.0 ng/ml for PGF₂α. In 4 of the 5 cows concentrations of PGE₂ were considerably lower than concentrations of PGF₂α. In the 2 cows without pyometra at slaughter, no fluid was present, and prostaglandin concentrations in the uterine flushings were 1.85 ± 0.7 for PGE₂ and 0.9 ± 0.2 for PGF₂α.
Detection of IFNα and IFNτ by Western Blotting

Representative results are shown in Fig 2. Multiple bands reacted nonspecifically with normal rabbit serum (NRS). Nonetheless, specific immunoreactive bands could be identified using both anti-rbIFNa and anti-rbIFNτ. For IFNα, the major immunoreactive band in lanes of rbIFNa was at 21 kDa and other bands were identified at 42 kDa and 10 kDa. For the lanes of rbIFNτ, anti-rbIFNτ reacted with a major band at 19 kDa and a minor band at ~40 kDa. Antisera for each interferon recognized both rbIFNa and rbIFNτ, with the greatest reactivity towards the interferon to which the antiserum was raised.

Specific bands corresponding to interferon-α were not detected in any sample of uterine fluid or flushings examined. There were some bands seen in some samples of pyometra fluid. These were either also seen in the lanes in which normal rabbit serum was used instead of antiserum or were yellow in color (as compared to the red reaction product) and were not considered to represent IFN-α. A 19 kDa band in pooled uterine flushings of pregnant cows reacted with antisera to rbIFNτ, but no specific immunoreactive bands were detected in the pool of uterine flushings from cyclic cows or from uterine secretions from any cow treated with A. pyogenes. To verify that uterine fluid from cows with pyometra did not interfere in the Western blotting procedures, samples of pooled pyometra fluid were blotted after addition of rbIFNa or rbIFNτ. In these lanes, an immunoreactive band for the relevant interferon could be identified when the blots were incubated with anti-rbIFNa or anti-rbIFNτ (results not shown).

DISCUSSION

Immune response to bacterial invasion involves infiltration of cellular components to the site of infection followed by production of various cytokines and prostaglandins (1, 3, 4, 21). Cytokines can alter the secretion of proteins and prostaglandins by a variety of tissues (1, 14, 27). In the uterus of the cow, prostaglandins are involved in both luteolysis and luteostasis. For example, PGF2α is luteolytic (26) and PGE2 exerts luteotropic actions (16, 32, 38). It was hypothesized in the present study that extended luteal function in cows with pyometra was caused by actions of leukocyte-derived IFNa, which can inhibit endometrial secretion of PGF2α (5, 31) and increase corpus luteum lifespan (29, 30, 31). While the present study provided evidence for pyometra-associated alterations in uterine PGE2 and PGF2α secretion, there was no evidence that these changes are caused by the production of IFNα.

It is unlikely that interferons are present in the uterus of pyometra cows at concentrations high enough to alter the secretion of PGF2α. Interferon-α was not detected by Western blotting in any of the uterine fluids or flushings from cyclic, pregnant or pyometric cows. The Western blotting system was capable of detecting IFNα in uterine flushings as indicated by the fact that IFNα was detected in flushings in which IFNα was added. It is possible that IFNα was present in uterine fluids at concentrations too low to be detected. If so, however, it is unlikely that such concentrations would have been high enough to extend the estrous cycle, since the Western blotting procedure was sensitive enough to detect antiluteolytic concentrations of IFNτ in the uterine flushes of pregnant cows. It is also possible that IFNα was produced earlier and had disappeared by Day 37. This seems unlikely because pyometra persisted through Day 37 in several cows. Furthermore, the patterns of PGE2 and PGF2α in the uterine fluids were different
Figure 2. Detection of interferons in uterine fluids by Western blotting. The arrow indicates immunoreactive interferons while the arrowhead identifies nonspecific bands found in uterine fluids and flushes that reacted with normal rabbit serum. The higher molecular weight bands seen in the sample of pyometra fluid were also either seen in some lanes in which normal rabbit serum was used instead of antiserum or were yellow in color (as compared to the red reaction product) and were not considered to represent IFN-α (color differences are not noticeable due to use of black and white photography). Data are representative of results using antiserum towards rbIFNa (top panel) or rbIFNγ (bottom panel). The NRS lanes are representative of samples of uterine fluid in which normal rabbit serum was used instead of antiserum.
than one would expect if IFNα extended the cycle since IFNα decreased secretion of PGE₂ and PGF₂α by bovine endometrial tissue (5).

The present finding that PGE₂ and PGF₂α are elevated in the uterine fluids of cows with pyometra is similar to that of previous reports of clinical cases of pyometra (18, 24). Prostaglandin F₂α predominated in the uterine fluids. Furthermore, cows with postpartum uterine infections have increased concentrations of the metabolite of PGF₂α (15 keto-13, 14-dihydroprostaglandin F₂α, PGFM) in peripheral blood plasma (12, 13, 33, 41, 39). The mechanism involved with the maintenance of the CL under conditions of increased endometrial PGF₂α are not yet fully understood. The persistent CL of pyometra may be a result of a lack of pulsatile PGF₂α release that normally occurs during luteolysis (28). Pregnant cows also have elevated concentrations of PGF₂α in uterine secretions (6, 17), and perhaps a common mechanism for CL refractoriness is involved in pyometra and pregnancy. Cows with pyometra had a lower concentration of plasma oxytocin than control cows after treatment with PGF₂α (40), and one possibility is that luteal receptors for PGF₂α are down-regulated as a result of exposure to high basal concentrations of prostaglandin. Another possibility is that PGE₂ counteracted the effects of PGF₂α since PGE₂ is luteotropic in the cow (16, 32, 38). Interleukin-1, a cytokine originally described in activated macrophages, can stimulate PGE₂ secretion from bovine endometrial epithelium and stroma (9, 11). Interleukin-1β has been identified in uterine flushings of the cow (9), but it is not clear if this cytokine is present in the uterine fluids of cows with pyometra. Enzyme-linked immunosorbent assay (ELISA) was used in the present study according to procedures described earlier (42) to measure IFNα, IFNγ, and interleukin-1β in uterine fluids. However, fluids from cows with pyometra interfered with the assay as indicated by low readings of pyometra fluids spiked with known amounts of cytokine (data not shown). It is likely that proteins in pyometra fluid inactivated antibody or antigen through proteolysis, or by masking of antigenic determinants or antibody binding sites.

The results of the present study confirm the original observation (10) that combined treatment of cows with hCG, iodine and live bacteria can induce pyometra; however, it is not clear whether or not the pathological changes caused by experimentally-induced pyometra mimicked these spontaneously-occurring pyometra. Findings such as endometrial abscesses were indicative of severe bacterial infection. In any case, induction of pyometra using procedures outlined here should prove useful in studying the causes of extended CL lifespan associated with pyometra. This is so because experimentally-induced pyometra altered CL lifespan in a manner similar to that seen in spontaneous pyometra. The increase in CL lifespan associated with pyometra in the present study was not a sole result of hCG or iodine administration; hCG given on Day 10 or 15 of the estrous cycle, and iodine given at Day 15 of the cycle extended CL lifespan in cows only by 4 to 7 days (25, 37, 43). Like cows with spontaneous pyometra, cows with induced pyometra had extended CL lifespan and increased concentrations of PGE₂ and PGF₂α in the uterine fluids. The physiological or immunological basis for these changes remains unclear, although IFNα production in the uterus is probably not involved.

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