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The effects of *A. pyogenes* on endometrial function *in vitro*, and on uterine and ovarian function *in vivo*

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Abstract

Uterine bacterial infection after parturition causes endometritis, perturbs ovarian function and leads to infertility in cattle. Although endometritis is caused by mixed infections, endometrial pathology is associated with the presence of *Arcanobacterium pyogenes*. The aims of the present study were to determine the effects of *A. pyogenes* on endometrial function *in vitro*, and on uterine and ovarian function *in vivo*. Heat-killed *A. pyogenes* did not affect the production of prostaglandin F_{2α} (PGF) or prostaglandin E₂ (PGE) from endometrial explants, or purified populations of endometrial epithelial or stromal cells. However, the explants produced more PGF and PGE than controls when treated with a bacteria-free filtrate (BFF) cultured from *A. pyogenes*. Similarly, BFF stimulated PGF and PGE production by epithelial and stromal cells, respectively. So, BFF or control PBS was infused into the uterus of heifers (n = 7 per group) for 8 days, starting the day after estrus. Emergence of the follicle wave, dominant follicle or corpus luteum diameter, and peripheral plasma FSH, LH, estradiol, progesterone, PGFM, or acute phase protein concentrations were unaffected by the BFF infusion. In the live animal it is likely that the intact uterine mucosa limits the exposure of the endometrial cells to the exotoxin of *A. pyogenes*, whereas the cells are readily exposed to the toxin *in vitro*.

Keywords

Inflammation; postpartum infection; reproductive immunology

Introduction

Bacterial contamination of the uterine lumen after parturition is common in dairy cattle, and uterine disease is an important cause of infertility. Within 2 weeks of parturition, 40% of cows develop metritis and infection persists in 15% of animals as endometritis (1,2). In typical studies of cows with endometritis compared with normal animals, conception rates

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are approximately 20% lower, the median calving to conception interval is 30 days longer and there are 3% more animals culled for failure to conceive (3,4). Furthermore, cows with a purulent cervical discharge have lower submission rates (5). In addition to the effects on reproduction, uterine disease causes pain, suffering and a systemic inflammatory response. For example, peripheral plasma concentrations of acute phase proteins such as haptoglobin or α_1 -acid glycoprotein (AGP) are increased in animals with uterine bacterial infection (6). Furthermore, after parturition plasma concentrations of the prostaglandin $F_{2\alpha}$ metabolite (13,14-dihydro-15-keto-PGF $_{2\alpha}$; PGFM) are increased, which likely reflect uterine tissue damage and infection (7,8).

The infertility associated with uterine disease is correlated with endometrial trauma and inflammation (9,10). However, there is also evidence of wider effects on the reproductive axis including persistence of the corpus luteum and failure of ovulation (11). The underlying mechanism for disruption of luteolysis is probably associated with bacteria stimulating production of more prostaglandin E_2 (PGE) than prostaglandin $F_{2\alpha}$ (PGF) from the endometrium (12). In addition, uterine disease suppresses the growth of ovarian dominant follicles and perturbs their function, as determined by lower peripheral plasma estradiol concentrations (2). Finally, bacteria, or their pathogen associated molecules, within the uterine lumen suppress the secretion of luteinising hormone (LH) from the pituitary (13,14); although the secretion of follicle stimulating hormone (FSH) appears to be unaffected (2).

In the postpartum cow, the pathogenic bacteria that are frequently isolated from cases of endometritis are *Escherichia coli* and *Arcanobacterium pyogenes* (15,16). The effects of *E. coli* or its pathogen-associated molecule, lipopolysaccharide (LPS), on reproductive function have been widely investigated (12-14). However, severe endometritis is particularly associated with the presence of *A. pyogenes* (10,15). *A. pyogenes* is a gram-positive aerobic bacillus that secretes its virulence factors rather than interacting directly with host cells (17). When infused into the uterine lumen of cows, a bacteria-free filtrate of *A. pyogenes* stimulates an immune response, as determined by an influx of neutrophils into the uterine lumen (18). However, the effects of *A. pyogenes* on uterine and ovarian function are less clear. The aims of the present study were to determine the effects of heat-killed *A. pyogenes* and a bacteria-free filtrate (BFF) on endometrial function *in vitro*, and on uterine and ovarian function *in vivo*.

Materials and Methods

Preparation of heat-killed *A. pyogenes* and a bacteria-free filtrate

A pathogenic strain of *A. pyogenes* was isolated from a postpartum cow showing clinical signs of endometritis subsequent to an episode of metritis with pyrexia (16). Swabs from the clinical case were cultured on sheep blood agar plates (Oxoid, Hants, UK) at 37°C for 48 h in an aerobic environment and preserved in semi-skimmed milk with 10% glycerol at -80°C. *A. pyogenes* was cultured from this bacterial stock on sheep blood agar plates and incubated at 37°C for 48 h. Colonies were sub-cultured in 600 mL Brain Heart Infusion broth (BHI; Oxoid) supplemented with 5% heat-inactivated foetal bovine serum (FBS; PAA, Somerset, UK) at 37°C for 48 h. The purity of the *A. pyogenes* broth and number of CFU/ml was determined by streaking 50 μ l onto sheep blood agar plates that were incubated for 48 h. Heat-killed *A. pyogenes* (HKAP) was prepared by heating half the *A. pyogenes* broth in BHI to 121°C for 15 min, followed by centrifuging at 12,000 $\times$ g for 12 minutes at 4°C. The supernatant was discarded and the pellet washed three times with ice-cold PBS; the bacteria were re-suspended in PBS and the HKAP stored at -20°C. The bacteria-free filtrate (BFF) was prepared by centrifugation of the remaining *A. pyogenes* broth at 12,000 $\times$ g for 12 min at 4°C. The supernatant was filtered through a 0.45 μ m durapore Millex-HV low protein-binding filter (Millipore, Billerica, USA) and the BFF stored at -80°C.

Endometrial explant and cell cultures

Uteri with no gross evidence of genital disease were collected from post-pubertal non-pregnant cattle at an abattoir immediately after slaughter and transported to the laboratory on ice. The physiological stage of the reproductive cycle for each genital tract was determined by observation of the ovarian morphology (19). Genital tracts with an ovarian Stage II corpus luteum were used because animals in the luteal phase are most susceptible to disease (20).

Tissue explants were established as previously described (12). Briefly, the endometrium was cut into strips and placed into RPMI-1640 medium (Sigma) supplemented with 50 IU/mL of penicillin, 50 µg/mL of streptomycin and 2.5 µg/mL of Amphotericin B (Sigma), working under sterile conditions. The strips were then chopped into 1 mm³ pieces using a mechanical chopper (McIlwain Laboratory Engineering, UK) and 50 mg of this tissue transferred onto metal grids in 6 well plates (Helena Biosciences, Switzerland) containing 4.25 mL of supplemented medium. The tissue explants were incubated at 37°C, 5% CO₂ in air, in a humidified incubator. After 24 h, the supernatants were discarded and replaced with 4.25 mL media containing 100 nM oxytocin (Bachem) and 100 µM aracadonic acid (Sigma) as a positive control to confirm physiological function, HKAP (10 to 10⁵ /mL), BHI (2 to 28% v/v) as a negative control for the vehicle containing BFF, BFF (equivalent to 0.4 × 10⁹ to 3.0 × 10⁹ CFU/mL), or 1 µg *E. coli* O55:B5 LPS (Sigma) as a positive control to confirm the uterine cells were capable of a pathophysiological response. The concentrations of HKAP and LPS were based on those used for heat-killed *E. coli* and LPS previously (12). Each experiment was performed in triplicate and repeated at least 3 times using explants established from separate animals. The supernatants were collected 24 h after treatment and stored at -20°C until analysed for prostaglandin concentrations.

For cell isolation, endometrial tissue was processed as previously described (12). Briefly, tissue was digested in sterile filtered digestive solution containing 50 mg trypsin III (Roche, Lewes, UK), 50 mg collagenase II (Sigma), 100 mg BSA (Sigma), and 10 mg DNase I (Sigma) in 100 mL HBSS in a shaking water bath at 37°C for 1.5 h. After incubation, the cell suspension was filtered through a 40 µm mesh (Fisher Scientific, Loughborough, UK) to remove undigested material, and the filtrate was resuspended in a HBSS washing medium containing 10% fetal bovine serum (FBS; Sigma) and 3 µg/mL trypsin inhibitor (Sigma). The suspension was centrifuged at 100 × *g* for 10 min and, after two further washes, the cells were resuspended in RPMI 1640 supplemented with 10% FBS, 50 IU/mL penicillin, 50 µg/mL streptomycin, and 2.5 µg/mL amphotericin B. The cells were plated in 24 well plates at a density of 1 × 10⁵ cells in 2 mL supplemented medium. To obtain separate stromal and epithelial cell populations, the cell suspension was removed 18 h after plating, which allowed selective attachment of stromal cells. The removed cell suspension was then plated in a 24 well plate and incubated to allow epithelial cells to adhere. Stromal and epithelial cell purity was greater than 95% determined by light microscopy and the production of prostaglandins E₂ and F_{2α} (21). All cultures were maintained at 37°C, 5% CO₂ in air, in a humidified incubator. The culture media was changed every 48 h until the cells reached confluence, and then treated with 100 nM oxytocin (Bachem) and 100 µM aracadonic acid (Sigma) as a positive control to confirm physiological function, BFF (equivalent to 0.8 × 10⁹ or 1.6 × 10⁹ CFU/mL) or 1 µg *E. coli* O55:B5 LPS as a positive control for a pathophysiological response. Each experiment was performed in triplicate and repeated at least 3 times using cells from separate animals. The supernatants were collected 24 h after treatment and stored at -20°C until analysed for prostaglandin concentrations.

Prostaglandin radioimmunoassay

Culture supernatants were analyzed for prostaglandin E₂ (PGE) and prostaglandin F_{2α} (PGF) by radioimmunoassay as previously described (22). Samples were diluted in 0.05 M Tris buffer containing 0.1% gelatin and 0.01% sodium azide. Standards and tritiated tracers for the prostaglandins were purchased from Sigma and Amersham International PLC (Amersham, Little Chalfont, UK), respectively. The antisera were a generous gift from Prof. N. L. Poyser (University of Edinburgh, UK) and their cross-reactivities were: PGF antiserum, 0.54% with PGE; PGE antiserum, 0.47% with PGF (23). The limits of detection for PGE and PGF were 2 pg/tube and 1 pg/tube, respectively. The intra- and inter-assay coefficients of variation were 4.4 and 7.8% for PGE, and 5.1 and 9.7% for PGF, respectively.

In vivo experiment

Seven nulliparous Holstein heifers aged between 18 to 20 months were assigned to control or BFF infusion in a crossover design. The animals received *ad lib* grass hay and 2 Kg/head concentrates twice daily (Growergrain Nuts, BOCM Pauls Ltd Ipswich), and were housed on straw in a naturally ventilated shed. No antimicrobial treatments were administered to heifers during the experiment. All procedures were carried out under Home Office authorisation in compliance with the Animals (Scientific Procedures) Act 1986 and ethical approval was granted from the Royal Veterinary College Ethical Review Committee.

Estrus was synchronised by administration of two intramuscular injections of a PGF analogue, cloprostenol (Estrumate, Schering-Plough Animal Health, Welwyn Garden City, UK) 11 days apart. The first injection (PG₁) ensured that a functional corpus luteum (CL) was present after 11 days. The second injection (PG₂) induced luteolysis of this CL to initiate ovulation, which was followed by a new wave of follicle growth and CL formation. Administration of a third prostaglandin injection (PG₃) 7 days after first observation of standing estrus induced luteolysis of the newly formed CL and subsequent ovulation of the expected dominant follicle. To confirm the sterility of uteri for heifers before treatments were administered, a transcervical-guarded swab was collected from the uterine body of each animal, before PG₁ was administered using a previously validated method (2). Each swab was placed in Stuart Transport Medium (Unipath, Basingstoke, UK), cultured aerobically and anaerobically, and bacteria identified using standard tests (2). In every case, the swabs were negative for pathogenic organisms.

Heifers were observed for signs of estrus for 30 min every 3 h for 3 days following the PG₂ injection. The onset of the estrous cycle was defined as the first time a heifer stood to be mounted by another heifer and this time was designated as Day 0. The stage of estrous cycle, and luteolysis after PG₂ was confirmed by measuring progesterone concentrations in peripheral plasma and ultrasound examination. Starting 24 h (designated Day 1) after first standing estrus was observed, the animals were randomly assigned to infusion of 10 mL BFF (equivalent to 1.6×10^{10} CFU/mL) or control infusion of 10 mL sterile PBS (Sigma), administered every 6 h for 9 days. The animals were monitored for signs of illness and the rectal temperature measured every 6 h for 9 days. Following a rest period of 21 days, all animals were subjected to the same protocol, but assigned to the alternate treatment group.

Ultrasound examination

To monitor ovarian function, transrectal ultrasonography using a 7.5 MHz linear array probe and a Honda HS-Transrectal 2000 machine (Honda Electronics, Aichi, Japan) was conducted every 12 h from the day before PG₂ administration for 14 days. Ovarian follicles were defined as non-echogenic black structures with clear demarcation between the follicular wall and stroma, whilst the CL was defined as an echogenic, grainy structure with

or without a non-echogenic lacuna. The diameter of follicles and CL were determined using the internal callipers of the ultrasonography machine by measuring the two dimensions of each structure at 90° angle and recording the average value. Ovarian follicles > 4 mm in diameter present in each ovary were identified, measured and recorded on an ovarian map. The emergence of a follicular wave was defined by the growth of a cohort of at least 2 follicles > 4 mm internal diameter and a dominant follicle was defined by an internal diameter > 8.5 mm in the absence of other growing follicles. The time of ovulation was retrospectively assigned as the day on which the dominant follicle disappeared from the ovary, if a CL subsequently developed in its place.

Blood Sampling

Blood samples were collected from the jugular vein into evacuated heparinised or plain tubes (BD Vacutainer Systems, Plymouth, UK). Blood samples were collected following administration of PG₂ every 3 h for six days, every 12 h for five days and finally every 3 h until the end of the study. Heparinised tubes were transported on ice and plasma separated within 1 h of collection by centrifugation at 1,600 × g for 10 min at 4°C. Blood samples collected in plain tubes were incubated at room temperature for at least 1 h then centrifuged at 1,600 × g for 10 min at 4°C to isolate serum. Isolated plasma and serum were stored at -20°C until assayed.

Hormone analysis

Once daily peripheral plasma progesterone concentrations were measured in duplicate using an enzyme linked immunoabsorbant assay, following the manufacturer's guidelines (Ridgeway Science, Gloucestershire, UK). It was assumed that a concentration of > 1 ng/mL indicated the presence of an active CL. The limit of detection for the assay was 0.05 ng/mL and the intra- and inter-assay coefficients of variation were 2.7% and 12.2%, respectively.

Once daily estradiol-17β concentrations were measured in peripheral plasma in duplicate, following diethyl ether extraction using the Estradiol MAIA radioimmunoassay kit (Serono Diagnostics Ltd; Woking, UK) as previously described (24). The limit of detection for the assay was 0.02 pg/mL and the inter- and intra-assay coefficients of variation were 20.8% and 21.6%, respectively.

The concentrations of LH and FSH were measured in duplicate in peripheral plasma as described previously (25). Standards and iodinated tracers were purchased from the National Hormone and Pituitary Programme (NHPP, California, USA) and Amersham International PLC (Amersham, UK), respectively. The internal recovery was 95% and the intra- and inter-assay coefficients of variation were 14.0% and 10.5% for FSH, and 14.0% and 10.4% for LH, respectively.

Once daily concentrations of PGFM were measured in peripheral plasma samples by radioimmunoassay as previously described (26). Standards and tritiated tracers were purchased from Sigma Aldrich and Amersham, and the PGFM antibody used was generously donated by H. Kindahl (Sweden). The limit of detection of the assay was 10 pg/mL, and the intra- and inter-assay coefficients of variation were 10.7 and 28.5%, respectively.

The concentration of the acute phase protein α₁-acid glycoprotein (AGP) was measured using a biochemical assay as previously described (6). The limit of detection for the assay was 0.05 ng/mL and the intra- and inter-assay coefficients of variation were 12.0% and 18.0%, respectively.

Insulin-Growth Factor 1 (IGF-1) concentrations were measured in peripheral plasma collected on Day 0 and 9. Samples were assayed in duplicate using a single Immunoassay kit according to the manufacturer's instructions (Immunodiagnostic systems; Tyne & Wear, UK). The limit of detection for the assay was 1.9 µg/L and the intra-assay coefficient of variation was 14.8%.

Statistical analysis

Data were normalised to when estrus was first observed and Day 1 was defined as the first day of intra-uterine infusion. Values are presented as the arithmetic mean $\pm$ SEM and significance was attributed when $P < 0.05$. Data from control and treated animals were compared by ANOVA and post hoc tests performed with Dunnett's adjustment, except for proportions, which were compared using the Fisher exact test.

Results

Endometrial tissue explants, epithelial and stromal cells

The endometrial explants produced more PGE (234.9 ± 13.2 vs. 33.9 ± 13.8 ng/mL, $P < 0.001$) and PGF (14.6 ± 1.4 vs. 3.6 ± 0.5 ng/mL, $P < 0.001$) in response to oxytocin and arachidonic acid than control tissues, confirming the tissues were physiologically functional *in vitro*. Stromal and epithelial cells have a polarised production of prostaglandin, producing predominantly PGE and PGF, respectively (12,21). When treated with oxytocin and arachidonic acid, the stromal cells produced more PGE (13.4 ± 0.7 vs. 0.7 ± 0.3 ng/mL, $P < 0.05$) and epithelial cells produced more PGF (168.0 ± 10.9 vs. 4.5 ± 0.4 ng/mL, $P < 0.001$) than controls, respectively. To ensure the growth medium for *A. pyogenes* did not stimulate prostaglandin production, the endometrial explants were treated with BHI. Addition of up to 28% BHI in the explant media did not significantly affect the production of PGF (4.5 ± 1.6 vs. 6.5 ± 2.2 ng/mL) or PGE (39.4 ± 6.2 vs. 42.4 ± 5.1 ng/mL) compared with controls.

The production of PGF from endometrial explants (Fig. 1a) or epithelial cells (Fig. 1b) treated with HKAP did not differ significantly from controls. Similarly, treatment with up to 10^5 HKAP did not significantly affect the production of PGE, compared with controls, from the explants (13.1 ± 2.6 vs. 9.3 ± 1.6 ng/ml) or stromal cells (21.8 ± 3.1 vs. 17.5 ± 2.6 ng/ml). However, endometrial explants treated with BFF produced more PGF (Fig. 2a) and PGE (Fig. 2b) than controls. Similarly, epithelial and stromal cells treated with BFF produced more PGF (Fig. 3a) and PGE (Fig. 3b) than controls, respectively. The endometrial explants, epithelial and stromal cells produced more prostaglandins in response to LPS (Fig. 2 and 3), which acted as a positive control in the present experiments to confirm the uterine tissues and cells were capable of responding to pathophysiological stimuli. In the light of the *in vitro* response to BFF but not HKAP, BFF was used for *in vivo* uterine infusion and the amount administered was based on the PGF and PGE response by the endometrial explants (Fig. 2).

In vivo experiment

The rectal temperatures of control and treated animals remained between 37.9 - 38.9 °C throughout the study and did not differ significantly between the treatment groups. Energy metabolism did not appear to be affected by BFF infusion as serum IGF-1 concentrations were similar for the control and treatment groups before (Day 0: 128.5 ± 6.9 vs. 129.9 ± 4.8 ng/mL) or after the infusions (Day 9: 131.7 ± 10.36 vs. 130.6 ± 7.7 ng/mL). Peripheral plasma PGFM concentrations did not differ between control and treatment groups before the first infusion was administered on Day 1 (20 ± 4 vs. 22 ± 2 pg/mL), Day 2 (20 ± 4 vs. 17 ± 4 pg/mL) or on Day 3 (26 ± 4 vs. 18 ± 2 pg/mL). Similarly, plasma concentrations of α_1 -AGP

did not differ significantly during the study between the control and treatment groups (1.35 ± 0.35 vs. 1.05 ± 0.18 ng/mL).

The time to LH peak from PG₂ was 61.3 ± 4.1 h and the peak LH concentrations were similar for control and treated animals (17.3 ± 3.3 vs. 15.8 ± 3.1 ng/mL). All animals ovulated after PG₂ as determined by the expression of estrus behaviour, ovarian ultrasonography and peripheral plasma progesterone concentrations < 1 ng/ml within 2 days. Following PG₂ a new wave of follicles emerged in all animals and a dominant follicle was identified for each heifer. The diameter of the dominant follicle and peripheral plasma estradiol concentrations increased with time ($P < 0.05$) but follicle growth and estradiol concentrations did not differ significantly between the control and treatment groups (Fig. 4a and b). Plasma FSH concentrations did not differ significant between the treatments groups (Fig 4c) and the LH concentration profiles were similar (Fig. 5). A new CL developed after PG₂ in all animals and peripheral plasma progesterone concentrations (Fig. 4d) or the diameter of the CL did not differ significantly between the control and treated animals (21.1 ± 0.6 vs. 18.1 ± 1.4 mm at the time of PG₃). Following PG₃, maximal peripheral plasma LH concentrations did not differ significantly between control and treated animals (13.7 ± 1.7 vs. 14.4 ± 2.7 ng/mL) and the time to LH peak was 51.6 ± 4.4 h. The proportion of animal ovulating did not differ between control and treated animals (6/7 vs. 5/7) and the maximum diameter of the ovulatory follicle did not differ significantly between the control and treated groups (13.7 ± 0.8 vs. 14.8 ± 1.0 mm).

Discussion

After parturition, infection of the uterus with *A. pyogenes* and *E. coli* is associated with clinical endometritis and infertility, and ovarian follicle growth and function are perturbed (1,27). Endometrial explants or cells produce PGE and PGF when treated with heat-killed *E. coli* or LPS (12). In the present study, treatment of endometrial explants or uterine epithelial and stromal cells with heat-killed *A. pyogenes* did not stimulate prostaglandin production. However, a bacteria-free filtrate (BFF) cultivated from *A. pyogenes* stimulated PGE and PGF production from endometrial explants, and from stromal and epithelial cells. These observations support the concept that the bacteria secrete a heat-labile toxin rather than interacting directly with host cells (28,29). However, when the same BFF was infused into the uterus of heifers there was no evidence of an effect on the immune response, as determined by acute phase protein or PGFM concentrations or rectal temperature, and there was no effect on their reproductive biology.

The cow provides a convenient model to study uterine disease because endometrium is readily available and pure populations of endometrial cells can be obtained (30). In the present study, the endometrial explants produced PGF and PGE in response to oxytocin and arachidonic acid, and the stromal and epithelial cells produced predominantly PGE and PGF, respectively, supporting previous observations (12,21). The explants and cells also produced prostaglandins in response to *E. coli* LPS, as reported previously (12). Thus, the uterine tissues and cells in the present study were capable of functional physiological and patho-physiological responses.

Heat killed *A. pyogenes* did not stimulate a prostaglandin response by the endometrial explants, epithelial or stromal cells. This is in marked contrast to the other major uterine pathogen, *E. coli*, where similar concentrations of heat killed bacteria stimulate substantial prostaglandin production and increased expression of gene transcripts for cytokines associated with inflammation (12). The explanation for the difference between the bacteria is likely to be that *E. coli* express LPS on their surface after heat-killing, which is highly immunogenic (31,32). In contrast, *A. pyogenes* appear to secrete a heat-labile haemolytic

exotoxin that is associated with tissue damage (28,29). Secretion of this exotoxin likely requires live bacteria or bacteria killed by a different method than heat and so a BFF was prepared from cultured *A. pyogenes*. Treatment of the endometrial explants or cells with BFF stimulated the production of PGE and PGF. This observation supports the concept that *A. pyogenes* exotoxin is likely to be more important during clinical uterine disease than direct interactions with the bacteria and so the effect of BFF was tested in the whole animal.

In the whole animal, infusion of *E. coli* or LPS into the uterus or peripheral blood perturbs ovarian follicular growth, ovulation, estradiol and LH secretion (13,14,33). The effects of *A. pyogenes* are less clear but infusion of BFF into the uterus provokes an influx of neutrophils into the uterine lumen (18,34). Furthermore, there was embryo mortality after uterine infusion of similar numbers of live *A. pyogenes* to the number of bacteria in the culture from which the BFF was derived (35). However in the present study, infusion of BFF into the uterine lumen of the heifers did not affect ovarian events, hormone concentrations, or markers of an inflammatory response such as PGFM and acute phase proteins. This might be because there was an insufficient concentration of toxin in the BFF, compared with that generated by live bacteria. Alternatively, heifers may be more resilient to uterine inflammation than postpartum cows, where the endometrium is already traumatised and there is lochia in which bacteria can readily proliferate, which would facilitate the growth and colonisation of *A. pyogenes*. Indeed, in cattle and sheep it was necessary to traumatise the endometrium to cause inflammation or establish experimental infections (36,37).

In conclusion, endometrial explants and purified populations of epithelial or stromal cells produced prostaglandins in response to BFF cultured from *A. pyogenes*, but not heat-killed bacteria. However, intrauterine infusion of BFF did not influence ovarian or endocrine events in heifers. In the live animal it is likely that the intact uterine mucosa is more resistant to the soluble exotoxin of *A. pyogenes* than explants or cells *in vitro*.

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Figure 1. The mean + SEM production of prostaglandin F_2 by endometrial (a) explants, and (b) epithelial cells treated with heat killed *A. pyogenes* (HKAP) at the concentrations indicated for 24 h.

Figure 2.

The mean + SEM production of (a) prostaglandin $F_{2\alpha}$ and (b) prostaglandin E_2 , by tissue explants treated for 24 h with BFF at the concentrations indicated or 1 $\mu\text{g/mL}$ *E. coli* LPS. Values differ significantly from control ***P < 0.01, **P < 0.05

Figure 3.

The mean + SEM production of (a) prostaglandin F₂ by endometrial epithelial cells, and (b) prostaglandin E₂ by stromal cells treated with BFF at the concentrations indicated or 1 µg/mL *E. coli* LPS. Values differ significantly from control ***P < 0.01, *P < 0.05

Figure 4.

The mean $\pm$ SEM (a) diameter of the dominant follicle and peripheral plasma concentrations of (b) estradiol, (c) FSH, and (d) progesterone, for of 7 heifers infused with control PBS (■) or BFF (●) for 8 days.

Figure 5. Representative peripheral plasma LH concentration profiles after PG₂ and PG₃ for animals that were infused with (a) control PBS, or (b) BFF.