Effect of seminal plasma or transforming growth factor on bovine endometrial cells

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Abstract

Semen induces post-coital inflammation of the endometrium in several species. Post-coital inflammation is proposed to alter the endometrial environment of early pregnancy, mediate embryonic development and modulate the maternal immune response to pregnancy. In cattle, it is common for pregnancies to occur in the absence of whole semen due to the high utilization of artificial insemination. Here, we have utilized a cell culture system to characterize semen-induced expression of inflammatory mediators in bovine endometrial cells and test the efficacy of transforming growth factor beta as the active agent in mediating any such change. We hypothesize that seminal plasma-derived transforming growth factor beta increases the expression of inflammatory mediators in bovine endometrial cells. Initially, we describe a heat-labile cytotoxic effect of seminal plasma on BEND cells, and a moderate increase in IL1B and IL6 expression. In addition, we show that transforming growth factor beta is present in bovine semen and can increase the expression of endometrial IL6, whereas blocking transforming growth factor beta in semen ameliorates this effect. However, intra-uterine infusion of seminal plasma, sperm or transforming growth factor beta did not alter the endometrial expression of inflammatory mediators. We conclude that bovine semen can modulate endometrial gene expression in vitro, which is partially due to the presence of transforming growth factor beta. It is likely that additional, unidentified, bioactive molecules in semen can alter the endometrial environment. Characterizing bioactive molecules in bovine semen may lead to the development of additives to improve artificial insemination in domestic species.

Introduction

Studies in mice, swine and humans have demonstrated that seminal plasma mediates a post-coital inflammatory response in female reproductive tissues which modulate the maternal response to pregnancy (Yanagimachi & Chang 1963, Robertson & Seamark 1992, O'Leary et al. 2004, Sharkey et al. 2012b). More recently, in vitro studies have confirmed a similar response in bovine endometrium (Ibrahim et al. 2019). The periconceptional period of pregnancy is a critical window of development that can influence embryo survival and affect the long-term health of offspring (Louis et al. 2008). In mice, pregnancies established in the absence of seminal plasma result in male offspring with altered metabolic phenotypes (Bromfield et al. 2014), suggesting that post-coital inflammation has long-term effects on pregnancy and offspring health.

Seminal plasma-derived transforming growth factor-beta (TGFβ) has been identified as a major component of semen to elicit the post-coital inflammatory response in female reproductive tissues of mice and humans (Tremellen et al. 1998, Robertson 2005), and its concentration in bovine seminal plasma is currently unknown. The endometrial inflammatory response to seminal plasma has been characterized in numerous species by increased expression of CSF2, IL1B, IL6, TNF, PTGS2, LIF and IL17A (Gutsche et al. 2003, O'Leary et al. 2004, Robertson et al. 2015). Many of these specific cytokines have the capacity to influence maternal immune cell populations during pregnancy (Chen et al. 2003, Ghiringhelli et al. 2005, Fridlender et al. 2009), in addition to acting as embryokines to support preimplantation embryo development (Hansen et al. 2014, Tribulo et al. 2018).

The success of assisted reproductive techniques including ICSI, IVF and artificial insemination affirms that seminal plasma is not essential for the establishment of pregnancy. Indeed, pregnancy rates using artificial insemination in cattle are similar to natural service (Lima et al. 2009), and generally pregnancy rates achieved by embryo transfer are higher than those for artificial insemination (Pellegrino et al. 2016). However, cows undergoing artificial insemination using diluted/extended semen are not exposed to physiological
components of the ejaculate. Hence, the response of the endometrium to semen is not well studied in cattle, while any influence of seminal plasma on bovine fertility is yet to be established. However, intrauterine infusion of TGFβ-1 at the time of artificial insemination increased pregnancy rates in cattle with low fertility (Odhiambo et al. 2009), while seminal plasma supplementation at the time of artificial insemination increased birth weight of heifer calves (Ortiz et al. 2019). In dairy cattle, the highest incidence of pregnancy loss occurs in the first week of pregnancy when approximately 40% of embryos fail to develop further than the blastocyst stage (Santos et al. 2004, Willbank et al. 2016). Therefore, this window of development is a critical target for modulating the maternal environment in order to increase pregnancy success in the cow. Previously, we have demonstrated that seminal plasma modulates the expression of inflammatory mediators in bovine ex vivo-cultured endometrial explants and semi-purified, primary endometrial cells (Ibrahim et al. 2019).

Here, we utilized in vitro culture of the bovine endometrial (BEND) cells and intrauterine infusion to evaluate cell viability and expression of cytokines and embryokines in response to semen components, including TGFβ. In addition, we have quantified the TGFβ content of bull semen. We hypothesize that seminal plasma or TGFβ increases the expression of cytokines and embryokines in endometrial cells under the influence of estradiol. The utility of the BEND cell line here could be used as a tool for future testing of paternal factors that may modulate endometrial function. The identification of semen components that could improve bovine reproduction and reduce economic losses associated with pregnancy failure may help to optimize artificial insemination in domestic species.

Materials and methods

Chemicals and reagents

All reagents were acquired from Fisher Scientific unless otherwise specified.

Seminal plasma collection

Whole semen was collected from healthy Angus bulls by electroejaculation during two separate breeding soundness evaluations (BSE) at the University of Florida North Florida Research and Extension Center. Whole semen from 21 bulls was collected on the first BSE and from 12 bulls were collected during the second BSE. Sample contamination was minimized as best could be achieved in field conditions. Whole semen was evaluated and further processed only if free of blood, urine and other visual abnormalities. Parameters recorded during the BSE included bull ID, collection date, ejaculated volume, scrotal circumference, and sperm motility, morphology and concentration. On average, whole semen volume ranged from 4 to 15 mL, gross motility ranged from 10 to 90%, and scrotal circumference ranged from 31 to 49 cm. Following semen collection, samples were placed on ice and transported to the laboratory for processing under aseptic conditions. Ejaculates were centrifuged at 12,000 g for 20 min at 4°C to separate seminal plasma from the cellular fraction of the ejaculate. Cell-free seminal plasma was placed in sterile tubes in aliquots of 500 to 1000 μL and stored at −20°C until use.

Preparation of pooled seminal plasma

Semenal plasma (1 or 2 mL) from 21 individual bulls, collected during the first BSE, was combined to prepare a seminal plasma pool. Pooled seminal plasma was either immediately stored at −20°C in 500 μL aliquots or filtered through a 0.22 μm filter prior to storage. In addition, thawed seminal plasma was also heat-treated at 90°C for 10 min prior application to cells. A second pool of seminal plasma was generated with ejaculates from 12 individual bulls of the second BSE.

Quantification of TGFβ in bull seminal plasma

Currently, there are no commercially available bovine-specific TGFβ ELISA kits and therefore a human TGFβ-1 and TGFβ-2 ELISA were used according to the manufacturer’s instructions (Human TFGβ-1 DuoSet, and Human TFGβ-2 DuoSet; R&D Systems). The concentrations of bioactive TGFβ-1 and TGFβ-2 were determined in untreated seminal plasma, while quantification of total TGFβ-1 and TGFβ-2 required acid activation of seminal plasma. For acid activation, 100 μL of seminal plasma was first diluted in 300 μL of Dulbecco’s phosphate buffered saline (DPBS) before the addition of 50 μL of 1 M HCl. Samples were incubated for 20 min at room temperature before neutralizing the pH with the addition of 50 μL of 1 M NaOH. Quantification of total TGFβ-1 and total TGFβ-2 was evaluated at 1:300 and 1:600, and 1:5 and 1:10 final dilutions respectively. Quantification of bioactive TGFβ-1 and TGFβ-2 were evaluated neat and at 1:5 and 1:10, respectively. The limit of detection for both TGFβ-1 and TGFβ-2 was 31.3 pg/mL. Both TGFβ-1 and TGFβ-2 ELISAs were validated using spike-in/recovery performance efficiency based on actual and expected recovery of recombinant TGFβ in each ELISA kit. The rate of recovery for TGFβ-1 was 82–97% and TGFβ-2 was 75–108%.

Reconstitution of rhTGFβ-1, rhTGFβ-2 and TGFβ pan specific antibody

A total of 10 μg of recombinant human transforming growth factor beta 1 (rhTGFβ-1) (240-B; R&D System) was reconstituted in 500 μL of sterile 4 mM HCl containing 1 mg/mL BSA fraction V in DPBS to achieve a 20 μg/mL stock solution. Stock solutions were diluted 1:20 in sterile DPBS containing 25 μg/mL of gentamicin to achieve a working stock concentration of 1 μg/mL rhTGFβ-1. Working stock solutions were stored in 40 μL aliquots at −80°C. A total of 5 μg of recombinant human transforming growth factor beta 2 (rhTGFβ-2) (302-B2; R&D System) was reconstituted in 50 μL of molecular grade water to achieve a 0.1 mg/mL stock solution. Stock solutions were diluted 1:100
in sterile DPBS containing 1 mg/mL BSA to achieve a working stock concentration of 1 μg/mL rhTGFβ-2. Working stock solutions were stored in 40 μL aliquots at −80°C.

A total of 1 mg of transforming growth factor beta pan specific antibody (αTGFβ) (AB-100-NA; R&D System) was reconstituted in 1 mL of sterile DPBS to achieve a 1 mg/mL stock solution. Working stock solutions were stored in 50 μL aliquots at −80°C. Prior to treatment, working stock solutions were diluted 1:20 in complete culture medium to achieve a concentration of 50 μg/mL of αTGFβ.

Culture of bovine endometrial (BEND) cells

Bovine endometrial epithelial (BEND) cells (Johnson et al. 1999) were purchased from American Type Culture Collection (ATCC) and cultured according to the distributor’s instructions. Briefly, BEND cells were cultured to 85% sub confluence in 75 cm² flasks (Greiner Bio-One, Frickenhausen, Germany) in a 1:1 mixture of Dulbecco’s Minimum Essential Medium (DMEM) and Ham’s F12 Nutrient Mixture Medium supplemented with 1.5 g/L sodium bicarbonate, 0.034 g/L L-α-valine, 10% fetal bovine serum (FBS), and 10% horse serum. Cultures were maintained at 38.5°C in a humidified 5% CO₂ environment. Complete culture medium was equilibrated to incubator conditions and changed every 48 h until cells reached sub confluence. Prior to treatment, cells were detached from flasks using 0.25% trypsin with 0.1% EDTA, washed in warm DPBS by centrifugation and resuspended in equilibrated culture medium. Cells were plated at a final concentration of 10⁵ cells/mL in 24- or 96-well culture plates (TPP, Trasadingen, Switzerland). Cultures were equilibrated for 24 h to facilitate cellular attachment before the addition of treatments (described below).

Isolation and culture of primary bovine endometrial epithelial and stromal cells

Uteri from post pubertal, non-pregnant cows were collected at a local slaughterhouse and processed at room temperature (RT) within 4 h of collection. Whole reproductive tracts were collected if no overt signs of infection were observed. A total of six reproductive tracts at stage 3 (day 11–17 of the estrous cycle) were used for all cultures. The stage of the estrous cycle was classified based on corpus luteum characteristics including internal and external appearance, diameter, surface vasculature, and presence of the corpus luteum as previously reported (Ireland et al. 1980).

Epithelial and stromal cells were isolated and cultured as previously described (Cronin et al. 2012). Briefly, external surfaces of the uteri were disinfected with 70% ethanol and the ipsilateral horn to the corpus luteum was opened with a transverse cut into the center of the horn, above the intercornual ligament and longitudinal to the ovary. The exposed endometrium was washed twice with DBPS supplemented with 50 IU/mL penicillin, 50 μg/mL streptomycin and 2.5 μg/mL amphotericin B. Endometrial tissue was further dissected into 3–5 mm² pieces and transferred into warm HBSS and maintained at 37°C for 10 min. Enzymatic digestion was achieved in HBSS supplemented with 100 mg BSA, 125 CDU/mg collagenase II (Sigma-Aldrich), 250 BAEE trypsin (Sigma-Aldrich), and 4% DNase I at 37°C with shaking for 1 h. Resultant cell suspension was then filtered through a 40 μm filter into warm HBSS supplemented with 10% FBS to stop enzymatic activity. The filtered suspension was centrifuged at 500 g for 7 min at RT and the endometrial cell pellet was resuspended in 5 mL of complete culture medium (RPMI 1640 medium supplemented with 10% FBS, 2 mM of L-glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin, and 2.5 μg/mL amphotericin B) equilibrated to 38.5°C. Cells were transferred to 75 cm² flasks (Greiner Bio-One) containing 25 mL of equilibrated culture medium and maintained at 38.5°C in a humidified 5% CO₂ environment. Following 18 h of culture, epithelial cells in suspension were transferred to a new flask while adherent stromal cells remained in the original culture flask. After a total of 66 h in culture, cells were detached from flasks using HyQase, washed with warm DPBS, resuspended at a final concentration of 10⁶ cells/mL and plated in 500 μL of culture medium in 24-well culture plates for 24 h prior to treatment. Purity of epithelial and stromal cells was assessed using flow cytometry and previously reported to be 56±16% and 95±3%, respectively (Ibrahim et al. 2019).

Treatment of primary and BEND cell cultures

All treatments were added to complete culture medium prior to the application to either BEND cells or primary endometrial cells. Treatments included medium alone as a negative control, seminal plasma (0.001%, 0.01%, 0.1%, 1%, 5%, 10%, or 20% v/v), rhTGFβ-1 (1 ng, 10 ng or 100 ng/mL), rhTGFβ-2 (0.1 ng, 1 ng or 10 ng/mL), or neutralizing pan-TGFβ antibody (1 μg, 5 μg, 10 μg, or 20 μg/mL). Seminal plasma was pre-incubated with neutralizing pan-TGFβ antibody for 5 min prior to application to cells. 17β-estradiol was supplemented to culture medium at a final concentration of 0, 0.1 or 1 nM. Cells were exposed to treatments for 0, 1, 3, 6, 12 or 24 h. Experiments using BEND cells were repeated at least four times and each replicate represents cells between passages 1 and 11. Experiments using primary cells were replicated six times, with each replicate being representative of endometrial cells from an individual cow. Following treatment, cells were washed with warm DPBS and stored in 350 μL of RLT Lysis Buffer (Qiagen) at −80°C to facilitate extraction of total RNA.

Evaluation of endometrial cell viability

Viability of BEND cells was assessed by colorimetric analysis using the MTT assay (Mosmann 1983). Cells were plated at 10⁵ cells/mL in 96-well culture plates (TPP) in a final volume of 200 μL of cell culture medium and equilibrated for 24 h prior to treatment. Treatments (above) were applied to cells in duplicate for 24 h and assays were repeated at least four times. Following treatment, 10 μL of 5 mg/mL MTT was added to each well and incubated for 2 h prior to washing in warm DPBS. Cellular formazan was solubilized in 100 μL of

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DMSO per well and incubated for 15 min at RT in the dark. Optical density of each well was measured at 540 nm using a microplate reader (BioTek, Winooski, VT, USA) and the blank corrected value for each treatment was calculated using the average of the duplicate wells. Data were normalized as fold change from the medium alone treated cells.

_Estrus synchronization, intrauterine infusion and endometrial sampling_

All animal use was approved and conducted in accordance to University of Florida Institutional Animal Care and Use Committee. Cows received 100 mg of GnRH (gonadorelin diacetate tetrahydrate; Ovacyst, Bayer Animal Health, Whippany, NJ, USA) i.m., followed 7 days later by 25 mg i.m. of prostaglandin F2α (dinoprost tromethamine; Prostamate, Bayer). Behavioral estrus was determined by activation of an estrus detection patch (ESTROTEC Breeding Indicator) applied at the time of prostaglandin administration. Intrauterine treatments were applied to cows 12 h following detection of behavioral estrus.

Treatments consisted of intrauterine infusion of either saline (0.9% NaCl; n = 5), washed sperm (n = 5), pooled seminal plasma (n = 5) or 10 ng rhTGFβ-1 (n = 5) applied using semen straws and artificial insemination technique. Sterile saline solution including 20 µL/mL antibiotic (Antibiotic Supplement for Neat Semen and CSS Extenders, Minitube, USA) was used as vehicle for all treatments. Pooled seminal plasma was packaged in 0.5 mL straws by Select Sires Inc. (Plain City, OH, USA) as previously described (Ortiz et al. 2019). Frozen, extended semen was thawed at 36.5°C for 30 s and centrifuged at 500 g for 5 min. The supernatant was discarded and washed sperm was resuspended in 0.5 mL vehicle solution and loaded into a single 0.5 mL straw for immediate intrauterine infusion. Sperm from a single bull was used throughout the experiment. All treatments were infused into the uterine body by standard artificial insemination technique (Ibrahim et al. 2019).

Endometrial cytobrush samples were collected 12 h after intrauterine infusion of treatments as previously described (Cardoso et al. 2017). Briefly, external genitalia were cleaned with 70% ethanol and the cytobrush tool (Medscand Medical, Cooper Surgical, Trumbull, CT, USA) which was held inside a metal sheath covered by a plastic chemise was introduced into the vagina and passed through the cervix by rectal palpation. The plastic chemise was retracted over the tool and the cytobrush was exposed to the endometrium and rotated three times to collect endometrial cells. Upon retraction, the cytobrush was snap frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from cytobrush samples using the TRIzol (Life Technologies, Carlsbad, CA, USA) method before processing for PCR (below).

_Results_

_The effect of seminal plasma on BEND cell viability_

Viability of BEND cells was assessed after 24 h exposure with medium alone or 0.001, 0.01, 0.1, 1, 5, 10, or 20% (v/v) seminal plasma (Fig. 1). To assess the cytotoxic effects of seminal plasma, pooled seminal plasma from the first BSE of 21 bulls was used. Viability of BEND cells was approximately 14% following exposure to 1% seminal plasma compared to medium alone (P ≤ 0.05), whereas viability was <2% following exposure to 5, 10 or 20% seminal plasma (Fig. 1A, P < 0.05). To determine the nature of the cytotoxic component of seminal plasma, treatments were either filtered, heat-treated or filtered and then heat-treated prior to exposure of cells. Filtering seminal plasma had no effect on seminal plasma cytotoxicity (Fig. 1B), whereas heat treatment reduced the cytotoxic effects of seminal plasma so that only 10 and 20% exposure resulted in cell death (Fig. 1C, P < 0.05). The combination of filtering and heat treatment of seminal plasma resulted in cytotoxicity...
Semen induced inflammation

The effect of seminal plasma on BEND cell gene expression

The capacity for seminal plasma to modulate BEND cell expression of CSF2, IL1B, IL6 and TNF was evaluated following 24-h exposure to 0.001, 0.01, 0.1, or 1% v/v seminal plasma or medium alone (Fig. 2). Seminal plasma from the first BSE of 21 bulls was either non-treated, filtered, heat-treated, or filtered and then heat-treated.

Expression of CSF2 (Fig. 2A) was unaffected following exposure to non-treated or filtered seminal plasma, whereas exposure to 0.1 or 1% (v/v) heat-treated or filtered and heat-treated seminal plasma reduced CSF2 expression compared to medium alone treated cells (P≤0.05). Expression of IL1B (Fig. 2B) was reduced following exposure to all seminal plasma treatment conditions compared to medium alone treated cells (P≤0.05). Specifically, IL6 expression was increased when exposed to 1% non-treated, filtered, or heat-treated seminal plasma when compared to medium alone (21.4-fold, 13.2-fold, and 1.6-fold, respectively; P≤0.05). Expression of TNF (Fig. 2D) was unaffected following exposure to seminal plasma compared to medium alone, with the exception of a 2.5-fold increase in expression following exposure to 0.001% heat-treated seminal plasma (P≤0.05).

The acute effect of seminal plasma on BEND cell expression

The effect of seminal plasma on BEND cell expression was evaluated after exposure to 1% v/v seminal plasma or medium alone for 0, 1, 3, 6 or 12 h (Fig. 3). The second pool of seminal plasma collected from 12 unique bulls was used during this and all subsequent experiments. Interestingly, the second pool of seminal plasma was cytotoxic at 5% v/v, whereas the first pool was cytotoxic at 1% v/v (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). Expression of CSF2 (Fig. 3A) or TNF (Fig. 3D) was unaffected following exposure to seminal plasma compared to control cells. Exposure to seminal plasma increased (P≤0.05) BEND cell expression of IL1B (Fig. 3B) compared to controls following 1, 3 or 6 h of treatment (increased 2.1-fold, 3.1-fold and 2.4-fold, respectively). Exposure to seminal plasma increased (P≤0.05) BEND cell expression of IL6 (Fig. 3C) compared to controls following 1, 3, 6, or 12 h of treatment (increased 4.1-fold, 3.9-fold, 2.5-fold and 5.9-fold, respectively).

The effect of estradiol supplementation on seminal plasma-mediated BEND cell function

To assess the capacity for estradiol to modulate seminal plasma-induced BEND cell gene expression, cells were exposed to medium alone or 0.001, 0.01, 0.1, or 1% v/v non-treated seminal plasma for 24 h in the presence of 0.1 nM or 1 nM estradiol (Fig. 4). Cells were supplemented with 0.1 nM or 1 nM of estradiol for 24 h prior to seminal plasma exposure. The addition of estradiol did not modulate the cytotoxic effects of seminal plasma when compared to estradiol-free cultures (Supplementary Fig. 1). Expression of CSF2 (Fig. 4A) or TNF (Fig. 4D) were unchanged following conditions compared to medium alone treated cells (P≤0.05). Specifically, IL6 expression was increased when exposed to 1% non-treated, filtered, or heat-treated seminal plasma when compared to medium alone (21.4-fold, 13.2-fold, and 1.6-fold, respectively; P≤0.05). Expression of TNF (Fig. 2D) was unaffected following exposure to seminal plasma compared to medium alone, with the exception of a 2.5-fold increase in expression following exposure to 0.001% heat-treated seminal plasma (P≤0.05).

Table 1  List of qPCR primer sequences for target gene analysis.

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer sequence</th>
<th>Reverse Primer sequence</th>
<th>GenBank accession number</th>
<th>Product size (bp)</th>
</tr>
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<tbody>
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<td>CSF2</td>
<td>TCTCGTGAAACCAAGTTATC</td>
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<td>NM_174027.2</td>
<td>144</td>
</tr>
<tr>
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<td>ATGGCAAGATGCTCACTTT</td>
<td>NM_001034034.2</td>
<td>114</td>
</tr>
<tr>
<td>IL1B</td>
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<td>CAGGTGTGATGCACTCTCT</td>
<td>NM_174093.1</td>
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</tr>
<tr>
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<td>180</td>
</tr>
<tr>
<td>TNF</td>
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<td>CTGGGGAAGCTGCTCTCTCT</td>
<td>NM_173966.3</td>
<td>261</td>
</tr>
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</table>
exposure to seminal plasma, regardless of estradiol treatment. Expression of IL1B (Fig. 4B) or IL6 (Fig. 4C) was increased ($P \leq 0.05$) following exposure to 1% seminal plasma compared to medium alone; however, this increase was not mediated by estradiol.

**Evaluation of transforming growth factor β content in bovine seminal plasma**

The content of TGFβ in seminal plasma was evaluated in 33 individual bulls (Supplementary Fig. 2). Total TGFβ-1 and TGFβ-2 was detectable at an average concentration of 7.11 ± 1.55 and 6.07 ± 1.16 ng/mL, respectively (range of 0.16–33.31 and 0.00–27.36 ng/mL, respectively). Bioactive TGFβ-1 and TGFβ-2 was detectable at an average concentration of 5.38 ± 0.88 and 331.09 ± 146.33 pg/mL, respectively (range of 0.00–28.56 and 0.00–4398.18 pg/mL, respectively). An average of 0.37 ± 0.12% of TGFβ-1 and an average of 4.15 ± 1.62% of TGFβ-2 was present in seminal plasma in the bioactive form. Using ejaculate volume and the concentration of total TGFβ, we calculated the absolute content of TGFβ-1 (63.4 ± 15.6 ng) and TGFβ-2 (58.2 ± 11.2 ng) in individual ejaculates.

**The effect of transforming growth factor β on BEND cell function**

The effect of rhTGFβ-1 (1, 10, or 100 ng/mL), or rhTGFβ-2 (0.1, 1, or 10 ng/mL) on BEND cell viability was assessed following 24-h exposure in medium supplemented with 0.1 or 1 nM estradiol. Regardless of estradiol supplementation, rhTGFβ-1 or rhTGFβ-2 had no effect on BEND cell viability compared to controls (Supplementary Fig. 3).

Expression of BEND cell CSF2, IL1B, IL6, and TNF was evaluated after 24-h exposure to rhTGFβ-1, rhTGFβ-2 or medium alone (Fig. 5). In the absence of estradiol, expression of TNF (Fig. 5D and H) was
increased following exposure to rhTGFβ-1 or rhTGFβ-2 compared to controls (P ≤ 0.05), but not when medium was supplemented with estradiol. Interestingly, in the presence of estradiol, BEND cell expression of IL6 (Fig. 5C and G) was increased following exposure to rhTGFβ-1 or rhTGFβ-2 compared to controls (P ≤ 0.05), but not when estradiol was absent. Exposure of BEND cells to rhTGFβ-1 had no effect on CSF2 or IL1B (Fig. 5A and B) expression regardless of estradiol supplementation. Conversely, expression of CSF2 and IL1B were reduced (P ≤ 0.05) following exposure to rhTGFβ-2 in the presence of 0.1 nM estradiol (Fig. 5E and F).

Figure 4 Effect of seminal plasma and estradiol on BEND cell gene expression. BEND cell expression of CSF2 (A), IL1B (B), IL6 (C), and TNF (D) was evaluated by qPCR. BEND cells were cultured in the presence of either 0, 0.1, or 1 nM estradiol for 24 h before the addition of seminal plasma (SP). BEND cells were then treated with 0.001, 0.01, 0.1, or 1 v/v SP for 24 h in medium supplemented with estradiol. Medium alone was used as a negative control. Data are presented as mean relative expression normalized to GAPDH using the 2^{-ΔΔCt} method ± s.e.m. Experiments were replicated in eight independent assays. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparisons were made between medium alone and each treatment. *, represents P ≤ 0.05 compared to medium alone.

Figure 5 Effect of transforming growth factor β on BEND cell gene expression. BEND cell expression of CSF2 (A and E), IL1B (B and F), IL6 (C and G), and TNF (D and H) was evaluated by qPCR. BEND cells were cultured in the presence of 0, 0.1 or 1 nM estradiol for 24 h before addition of rhTGFβ. BEND cells were then exposed to either rhTGFβ-1 (0, 1, 10 or 100 ng/mL; A, B, C, D), or rhTGFβ-2 (0.1, 1 or 10 ng/mL; E, F, G, H) for 24 h in medium supplemented with estradiol. Data are presented as mean relative expression normalized to GAPDH using the 2^{-ΔΔCt} method ± s.e.m. Experiments were replicated in at least four independent assays. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparisons were made with the 0 ng/mL control within each estradiol concentration. *, represents P ≤ 0.05 compared to appropriate control within an estradiol concentration.
The acute response of BEND cells to rhTGFβ-1 (100 ng/mL), or rhTGFβ-2 (10 ng/mL) was evaluated following exposure for 0, 1, 3, 6, or 12 h in medium supplemented with 0.1 nM estradiol (Fig. 6). Exposure to rhTGFβ-1 or rhTGFβ-2 had no effect on BEND expression of CSF2, IL1B or TNF at any time point (Fig. 6). However, expression of IL6 was increased ($P \leq 0.05$) after exposure to rhTGFβ-1 or rhTGFβ-2 for 3, 6 and 12 h compared to controls (Fig. 6C and G).

**Effect of neutralizing seminal plasma transforming growth factor β on BEND cell gene expression**

To determine the degree to which seminal plasma TGFβ modulates BEND cell gene expression, seminal plasma was pre-incubated with TGFβ neutralizing antibody ($\alpha$TGFβ) prior to application to BEND cells. Expression of CSF2, IL1B, IL6 and TNF was evaluated by qPCR following 24-h treatment (Fig. 7). All cells were cultured in the presence of 0.1 nM estradiol before and during treatments.

Seminal plasma induced a 28.4-fold increase in BEND cell IL6 expression (Fig. 7C). The addition of 20 μg/mL of $\alpha$TGFβ inhibited seminal plasma induced IL6 expression by 49.5% compared to cells exposed to seminal plasma alone ($P \leq 0.05$). Seminal plasma induced a 9.3-fold increase in IL1B expression ($P \leq 0.05$) which was not affected by the addition of $\alpha$TGFβ (Fig. 7B). As previous, seminal plasma did not alter BEND cell expression of CSF2 or TNF (Fig. 7A and D).

**Effect of seminal plasma or transforming growth factor β on primary endometrial stromal and epithelial cell gene expression**

The capacity of seminal plasma or TGFβ to modulate expression of CSF2, IL1B, IL6 or TNF was assessed in primary bovine endometrial stroma (Fig. 8A, B, C and D) or epithelial cells (Fig. 8E, F, G and H). Cells were exposed to seminal plasma (0.001, 0.01, 0.1, or 1% v/v), rhTGFβ-1 (1, 10 or 100 ng/mL), rhTGFβ-2 (0.1, 1 or 10 ng/mL), or medium alone for 24 h. Stromal cell expression of CSF2, IL6 and TNF were increased ($P \leq 0.05$) following exposure to 1% seminal plasma compared to control cells (11.0-fold, 28.4-fold and 63.1-fold, respectively). Seminal plasma had no effect on epithelial cell gene expression. Exposure to rhTGFβ-1 or rhTGFβ-2 had no effect on either endometrial stromal or epithelial cells.
Semen induced inflammation

Effect of intrauterine infusion of semen components on endometrial gene expression

Endometrial response to saline, washed sperm, seminal plasma or rhTGFβ-1 was evaluated 12 h following intrauterine infusion (Fig. 9). The average number of lactations and the average number of days postpartum at the time of infusion was similar among treatment groups (2.4 ± 0.2 lactations and 448 ± 24.4 days postpartum; P > 0.05). Endometrial expression of CSF2, IL1B, IL6 and TNF was unchanged (P > 0.05) following intrauterine infusion of any treatment.

Discussion

Since the 1960s, post-coital inflammation has been characterized in rodents, humans, horse, and swine (Yanagimachi & Chang 1963, O et al. 1988, Robertson & Seamark 1992, Troedsson et al. 2001, O’Leary et al. 2004, Sharkey et al. 2007). Our own work demonstrates that seminal plasma increases the expression of inflammatory mediators in bovine primary cultures (Ibrahim et al. 2019). Reports from mice have shown that seminal plasma exposure at coitus is associated with modulating pregnancy outcomes and subsequent offspring performance (Bromfield et al. 2014). Initial investigations into post-coital endometrial inflammation defined seminal vesicle gland-derived TGFβ as the active moiety responsible for maternal inflammation at the time of insemination (Robertson et al. 1997). Due to the increased utilization of artificial insemination in the cattle industry, we proposed to further investigate

Figure 7 Effects of neutralizing transforming growth factor β antibody on seminal plasma induced gene expression. Expression of CSF2 (A), IL1B (B), IL6 (C), and TNF (D) was evaluated by qPCR. BEND cells were cultured in the presence of 0.1 nM estradiol for 24 h before addition of treatments. BEND cells were then treated with medium alone or 1% v/v seminal plasma (SP) in the presence of pan-TGFβ neutralizing antibody. Treatments were pre-incubated with 1, 5, 10, or 20 μg/mL of antibody for 5 min prior to exposure of cells for 24 h. Data are presented as the mean relative expression normalized to GAPDH using the 2^ΔCt method ± S.E.M. Experiments were replicated in six independent assays. Data were analyzed using the generalized linear mixed model and pairwise comparisons were made between SP and medium alone within a given antibody concentration. *, represents P ≤ 0.05.

Figure 8 Effect of seminal plasma or transforming growth factor β on primary endometrial cell gene expression. Expression of CSF2, IL1B, IL6 and TNF was evaluated in primary endometrial stromal (A, B, C and D) or epithelial (E, F, G and H) cells after 24-h treatment with either seminal plasma (SP; 0.001, 0.01, 0.1, or 1% v/v), rhTGFβ-1 (1, 10 or 100 ng/mL), rhTGFβ-2 (0.1, 1 or 10 ng/mL) or medium alone. Data are presented as the mean relative expression normalized to GAPDH using the 2^ΔCt method ± S.E.M. Experiments include six independent assays with each replicate of cells obtained from an individual cow. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparisons were made between medium alone and each treatment. *, represents P ≤ 0.05 compared to medium alone.

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the role of semen components in modulating the postcoital maternal environment. Using the cell line, BEND cells, we initially observed a heat-labile cytotoxic effect of seminal plasma starting from 1% v/v. Subsequently, we observed a seminal plasma-induced increase in *IL1B* and *IL6* BEND cell expression which was independent of estradiol exposure. In addition, we tested the capacity of TGFβ to elicit endometrial change, as observed in other species. While seminal plasma increased expression of *CSF2, IL6* and *TNF* in primary endometrial cells, TGFβ had no effect. However, both TGFβ-1 and TGFβ-2 increased BEND cell expression of *IL6* in an estradiol-dependent manner, while *TNF* was increased only in the absence of estradiol. Blocking seminal plasma TGFβ reduced the capacity of BEND cells to increase *IL6* expression but did not inhibit expression entirely. Interestingly, we did not observe any effect on endometrial gene expression following intrauterine infusion of seminal components.

The natural site of semen deposition in cattle is the fornix vagina and sperm travel through the cervix into the uterus, while most seminal plasma remains in the vagina and is lost through retrograde transport. It is unclear how much, if any, seminal plasma is delivered into the uterus in cattle, whereas horses and swine receive a large dose of seminal plasma directly into the uterine lumen. These anatomical differences of conception could influence the dynamics by which the female reproductive tract communicates with semen components. However, artificial insemination bypasses the cervix and results in extended semen being delivered directly into the uterus. Current semen extension protocols result in commercial semen doses containing variable concentrations of seminal plasma (Bergeron et al. 2004). In this regard caution must be applied when interpreting the results of the above studies when extrapolating to the physiological response to natural insemination. The studies described herein bypass the physiological site of semen deposition which may be important, or perhaps endometrial response requires simultaneous exposure to both seminal plasma and sperm to elicit endometrial responses? We are relatively confident that the timing of exposure to semen components was optimal given that cows were observed at estrus when circulating estrogen is maximal and has been previously described as being critical for seminal plasma elicited responses in the endometrium of rodents (Robertson & Seamark 1990).

However, future experimental approaches should utilize a time course approach to ensure that any semen-mediated response is evaluated in the endometrium; it may be possible that a 12-h delay between treatment and sampling is too protracted to evaluate any response. Although our previous intra-uterine infusion of seminal plasma suggests that an endometrial response was apparent at 24 h post infusion (Ibrahim et al. 2019).

There are several points at which pregnancy can fail. Elegant studies in cattle suggest that 40% of pregnancy failure occurs within 1 week of conception (Santos et al. 2004, Wiltbank et al. 2016). This suggests that early pregnancy loss is a result of fertilization errors, embryonic failure, endometrial failure or ovarian insufficiency; theoretically, seminal plasma could influence these factors based on observations made in other species. In rodents, seminal plasma increases the expression of endometrial embryokines expression to support preimplantation embryo development, while modulating maternal immune cell populations to facilitate the implantation and invasion of the semi-allogeneic conceptus. In rodents and swine, seminal plasma exposure mediates ovarian function by increasing progesterone synthesis or altering immune cells within the corpus luteum (Gangnuss et al. 2004, O’Leary et al. 2002, 2006). Unprotected intercourse at the time of embryo transfer, or seminal plasma exposure during IVF treatment increases clinical pregnancy rates in women (Tremellen et al. 2000, Saccone et al. 2019). It is unclear if seminal plasma can facilitate the same effect on pregnancy success in cattle. However, we have demonstrated that supplementation of seminal plasma at the time of artificial insemination increases birth weight of heifer calves, but does not influence conception rates (Ortiz et al. 2019). Our previous in vitro data suggest that seminal plasma has the potential to increase the expression of embryokines such as *CSF2* and *IL6* to support the preimplantation bovine embryo (de Moraes & Hansen 1997, Ibrahim et al. 2019, Wooldridge & Ealy 2019). Identification of factors in seminal plasma, such as TGFβ, which could modulate these key time points in pregnancy may lead to intervention strategies that could
be supplemented at artificial insemination and improve pregnancy outcomes in cattle. Murine seminal plasma contains predominately TGFβ-1 and TGFβ-2 isoforms and elicits endometrial inflammation similar to that induced by seminal plasma exposure (Robertson 2005). TGFβ-1, TGFβ-2 and TGFβ-3 are plentiful in human seminal plasma and stimulate inflammatory changes in ectocervical epithelial cells, mimicking the post coital uterine environment (Sharkey et al. 2012a). We found that bull seminal plasma contains TGFβ-1 and TGFβ-2 in both active and latent forms. Following insemination in mice, 70% of TGFβ in uterine fluid is present in the active form, suggesting that activation of TGFβ occurs at the time of ejaculation or after deposition in the female tract (Tremellen et al. 1998). In human, the acidic environment of the vagina (pH 5.0) could be enough to remove TGF-binding protein and activate latent protein. Latent seminal plasma TGFβ may also involve seminal plasma enzymes, including plasmin, subtilisin-like endoproteases and urokinase type plasminogen activator (Chu & Kawinski 1998, Robertson et al. 2002). In regard to the content of TGFβ comparisons to other species can be confounded by ejaculate volume and site of semen deposition. Here we report the absolute content of bull semen TGFβ-1 and TGFβ-2 as 63 ng and 58 ng, respectively. Comparatively, human seminal plasma contains 220–240 ng/mL and 5–18 ng/mL of TGFβ-1 and TGFβ-2 (Nocera & Chu 1995), while mouse and swine semen TGFβ-1 content has been estimated at 74 ng/mL and 150 ng/mL, respectively (Tremellen et al. 1998, O’Leary et al. 2011). Interestingly, neutralizing seminal plasma TGFβ by use of a pan-specific antibody, mediated a moderate reduction in IL6 gene expression, future experiments should clarify this response using inhibitors of TGFβ signaling or increased incubation time of seminal plasma with blocking antibodies. Additionally, future experiments should evaluate the secretion of bioactive molecules into cell culture supernatants and uterine fluid following treatment with semen components.

Here bovine seminal plasma has a heat-labile cytotoxic effect on BEND cells, implying that cytotoxicity is protein mediated. Cytotoxicity of bovine seminal plasma has been previously reported at high concentrations (4% v/v) in primary endometrial cells and is associated with fertility, with low fertility bulls increasing cytotoxicity (Nongbua et al. 2018). In addition, it has been reported that the protein content of semen is associated with pregnancy rate, with low protein abundance associated with low conception rate (Moura et al. 2006). Unique to the bull is the expression of bovine seminal RNase (Jermann et al. 1995). In addition to the immunosuppressive function of bovine seminal RNase (Tamburrini et al. 1990), this enzyme has been shown to have direct cytotoxic activity (Matousek et al. 2003, Viola et al. 2005). Interestingly, we observed that the cytotoxic potential of seminal plasma varied between the two unique populations of bulls used in the current study, with the first population of bulls exhibiting toxicity at lower concentrations than the second population of bulls. This effect may be due to variability in semen constituents between individuals driven by genetic variability, season of collection, feed, exposure to toxins, presence of bacterial toxins or bull age. Future studies should utilize seminal plasma from individual bulls to isolate the cytotoxic components of semen; seminal plasma cytotoxicity may be associated with bull fertility.

Previous analysis of bull semen reports that osteopontin and lipocalin-type prostaglandin D synthase content of seminal plasma is positively associated with increased bull fertility (Cancel et al. 1997, Gerena et al. 1998). The role for either protein is currently unknown but may play a role in sperm function or sperm-uterine interactions. Conversely, seminal plasma derived TGFβ is the active molecule in semen to drive postcoital inflammation and maternal cytokine expression in rodents and humans (Tremellen et al. 1998, Sharkey et al. 2012a). Intrauterine supplementation with 40 ng of rhTGFβ-1 at the time of artificial insemination increased conception rates in beef cattle herds with poor fertility but had no effect on herds when conception was above 50% (Odhiambo et al. 2009).

Our data indicate that seminal plasma-mediated increased expression of BEND cell IL1B and IL6 is independent of estradiol, while TGFβ-1 or TGFβ-2 increased BEND cell IL6 expression in an estradiol-dependent manner. Conversely, neither TGFβ-1 nor TGFβ-2 increased expression of any cytokine measured in primary cells. These discrepancies may be a direct reflection of the cell types used in each experiment. BEND cells were isolated from cows at day 14 of the estrous cycle and are presumed to be positive for bovine diarrheal virus (Johnson et al. 1999), whereas primary endometrial cells cultured here were isolated at days 11–17 of the estrous cycle based on ovarian morphology. The responsiveness of these two cell types to estrogen is questionable, as is their capacity to respond to human recombinant TGFβ protein. Additionally, the transformation of BEND cells or long-term culture may impair responsiveness to a degree which does not recapitulate primary isolated cells which would be exposed to semen components at insemination under the same endocrine environment. Interestingly, we found that the cytotoxicity of seminal plasma varied between batches, and it is very likely that responsiveness of cells to seminal plasma may change due to the presence or absence of specific bulls within the sampled population. Interestingly, our previous data reported that seminal plasma increased endometrial expression of various inflammatory cytokines that was only mildly recapitulated in these current studies with primary cells. The source of seminal plasma is likely the root cause of this discrepancy and may require the testing of individual bulls for endometrial responsiveness.
The utility of artificial reproductive technologies clearly demonstrates that seminal plasma is not a requirement for pregnancy. However, work in various other species suggests that paternal factors can modulate endometrial function and potentially influence pregnancy outcomes. The heavy use of artificial insemination in the cattle industry, where insemination occurs in the absence of (or very dilute) seminal plasma, may provide an opportunity to improve pregnancy outcomes by simple supplementation of paternal factors at the time of insemination. These studies begin to describe paternal seminal factors that could be tested in cell lines and potentially targeted for further investigation to improve reproductive performance of cattle.

**Supplementary data**

This is linked to the online version of the paper at [https://doi.org/10.1530/REP-19-0421](https://doi.org/10.1530/REP-19-0421).

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Author contribution statement**


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**References**

Bergeron A, Crete MH, Brindle Y & Manjunath P 2004 Low-density lipoprotein fraction from hen’s egg yolk decreases the binding of the major proteins of bovine seminal plasma to sperm and prevents lipid efflux from the sperm membrane. *Biology of Reproduction* 70 708–717. [https://doi.org/10.1095/biolreprod.104.029996](https://doi.org/10.1095/biolreprod.104.029996)

Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ & Robertson SA 2014 Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *PNAS* 111 2200–2205. [https://doi.org/10.1073/pnas.1305609111](https://doi.org/10.1073/pnas.1305609111)


Cronin JG, Turner ML, Goetzl L, Bryant CE & Sheldon IM 2012 Toll-like receptor 4 and MYD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. *Biology of Reproduction* 86 51. [https://doi.org/10.1095/biolreprod.111.092718](https://doi.org/10.1095/biolreprod.111.092718)


Hansen PJ, Dobbs KB & Denicol AC 2014 Programming of the preimplantation embryo by the embryokine colony stimulating factor 2. *Animal Reproduction Science* 149 59–66. [https://doi.org/10.1016/j.aniproc.2014.05.017](https://doi.org/10.1016/j.aniproc.2014.05.017)

Ibrahim LA, Rizo JA, Fontes PLP, Lamb GC & Bromfield JJ 2017 Seminal plasma modulates expression of endometrial inflammatory mediators in the bovine†. *Biology of Reproduction* 100 660–671. [https://doi.org/10.1086/683198](https://doi.org/10.1086/683198)


Jermann TM, Optiz JG, Stockhouse J & Benner SA 1995 Reconstructing the evolutionary history of the antioductal ribonuclease superfamily. *Nature* 374 57–59. [https://doi.org/10.1038/374057a0](https://doi.org/10.1038/374057a0)

Johnson GA, Austin KJ, Collins AM, Murdoch WJ & Hansen TR 1999 Endometrial ISG17 mRNA and a related mRNA are induced by interferon-τ and localized to glandular epithelial and stromal cells from pregnant cows. *Endocrine* 10 243–252. [https://doi.org/10.1007/BF02738623](https://doi.org/10.1007/BF02738623)


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Semen induced inflammation


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