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.

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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 than 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 (TGF β) 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 TGF β -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, Wiltbank *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 TGF β . In addition, we have quantified the TGF β content of bull semen. We hypothesize that seminal plasma or TGF β 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 μ L and stored at –20°C until use.

Preparation of pooled seminal plasma

Seminal 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 μ L aliquots or filtered through a 0.22 μ m 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 TGF β in bull seminal plasma

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

Reconstitution of rhTGF β -1, rhTGF β -2 and TGF β pan specific antibody

A total of 10 μ g of recombinant human transforming growth factor beta 1 (rhTGF β -1) (240-B; R&D System) was reconstituted in 500 μ L of sterile 4 mM HCl containing 1 mg/mL BSA fraction V in DPBS to achieve a 20 μ g/mL stock solution. Stock solutions were diluted 1:20 in sterile DPBS containing 25 μ g/mL of gentamicin to achieve a working stock concentration of 1 μ g/mL rhTGF β -1. Working stock solutions were stored in 40 μ L aliquots at –80°C.

A total of 5 μ g of recombinant human transforming growth factor beta 2 (rhTGF β -2) (302-B2; R&D System) was reconstituted in 50 μ L 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 D-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 to remove mucus and potential microorganisms. Endometrial tissue from intercaruncular regions was dissected into thin strips and placed into Hank's balanced salt solution (HBSS) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin and 250 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 BAE 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 HyQTase, washed with warm DPBS, resuspended at a final concentration of 10⁵ 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

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 F_{2α} (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. Semen 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).

Extraction of cellular RNA and qPCR

Total RNA was extracted from cultured cells using the RNeasy Mini Kit according to manufacturer's instructions (Qiagen).

Extracted RNA was resuspended in 30 µL of RNAase-free water and quantified using a NanoDrop ND1000 spectrophotometer. A total of 1 µg of RNA was subjected to RT

using the Verso cDNA synthesis kit according to manufacturer's instructions including a genomic DNA wipe out step. Resultant cDNA was diluted 1:3 in molecular grade water and stored at -80°C.

Primers used for real-time RT-PCR were designed using the NCBI database and verified by BLAST. Primer details are listed in Table 1. Amplification efficiency was evaluated for each primer by performing serial dilutions of cDNA to confirm linear amplification and confirmed by visualization of PCR product on a 1% agarose gel. All primers met MIQE guidelines for use (Pearson correlation coefficient $r^2 > 0.98$ and efficiency between 90 and 110%). Real-time RT-PCR was performed in 20 µL reactions using iTaq Universal SYBR green chemistry (Bio-Rad) with 100 nM of each forward and reverse primer. A Bio-Rad CFX Connect light cycler (Bio-Rad) was employed to perform PCR using a two-step protocol with the following recommended thermal cycling conditions: initial denaturation/enzyme activation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, and annealing/extension for 30 s at 60°C. Samples were analyzed in duplicate and each PCR reaction was followed by melt curve analysis to ensure single product amplification. A no template negative control was used in place of cDNA to determine non-specific amplification. Expression data for genes of interest were normalized to *GAPDH* and expressed using the $2^{-\Delta Ct}$ method.

Statistical analysis

Data were analyzed using SPSS software V24.0 (IBM Analytics, Armonk, NY, USA). Data were assessed with Shapiro-Wilk test and log transformed when appropriate (described in figure legends). Data were analyzed using the generalized linear mixed model with dose, treatment and time as fixed factors. Pairwise comparisons were performed between each dose and vehicle controls. Statistical significance was declared when $P \leq 0.05$.

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 ($P \leq 0.05$), 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

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

Gene	Primer sequence (5'–3')		GenBank accession number	Product size (bp)
	Forward	Reverse		
<i>CSF2</i>	TTCCTGTGGAACCCAGTTTATC	TTTGGCCTGCTTCACTTCT	NM_174027.2	114
<i>GAPDH</i>	AGGTCGGAGTGAACGGATTC	ATGGCGACGATGTCCACTTT	NM_001034034.2	114
<i>IL1B</i>	CTTCATTGCCAGGTTTCTG	CAGGTGTTGGATGCAGCTCT	NM_174093.1	142
<i>IL6</i>	ATGACTTCTGCTTCCCTACCC	GCTGCTTTCACACTCATCATT	XM_015468553.1	180
<i>TNF</i>	CACATACCCTGCCACAAGGC	CTGGGGACTGCTCTTCCCTCT	NM_173966.3	261

effects following exposure to 10 or 20% seminal plasma (Fig. 1D, $P < 0.05$). Seminal plasma was not toxic to BEND cells at concentrations between 0.001 and 0.1%.

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 ($P \leq 0.05$). Expression of *IL1B* (Fig. 2B) was reduced following exposure to all seminal plasma treatment conditions compared to medium alone treated cells ($P \leq 0.05$). Expression of *IL6* (Fig. 2C) was increased following exposure to all seminal plasma treatment

conditions compared to medium alone treated cells ($P \leq 0.05$). 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; $P \leq 0.05$). 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 ($P \leq 0.05$).

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 ($P \leq 0.05$) 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 ($P \leq 0.05$) 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

Figure 1 Effect of seminal plasma on BEND cell viability. Cell viability was assessed using the MTT assay after 24-h treatment with medium alone (0) or 0.001, 0.01, 0.1, 1, 5, 10, or 20% v/v seminal plasma (SP). Seminal plasma was either non-treated (A), filtered (B), heat-treated (C) or both filtered and heat-treated (D). Data are presented as mean fold-change of medium alone treated cells $\pm$ S.E.M. Experiments were replicated in at least 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 seminal plasma concentration. *, represents $P \leq 0.05$ compared to medium alone.

Figure 2 Effect of seminal plasma on BEND cell gene expression. BEND cell expression of *CSF2* (A), *IL1B* (B), *IL6* (C), and *TNF* (D) was evaluated by qPCR after 24-h treatment with medium alone (0) or 0.001, 0.01, 0.1, or 1% v/v seminal plasma (SP). Seminal plasma was either non-treated, filtered, heat-treated, or filtered and heat-treated. Data are presented as mean relative expression normalized to *GAPDH* using the $2^{-\Delta Ct}$ method $\pm$ S.E.M. Experiments were replicated in seven independent assays. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparisons were made between medium alone and each seminal plasma concentration. *, represents $P \leq 0.05$ compared to medium alone.

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.

Figure 3 Acute effects of seminal plasma on BEND cell expression. BEND cell expression of *CSF2* (A), *IL1B* (B), *IL6* (C), and *TNF* (D) was evaluated following treatment with medium alone or 1% v/v seminal plasma (SP) for 0, 1, 3, 6 or 12 h. Data are presented as the mean relative expression normalized to *GAPDH* using the $2^{-\Delta Ct}$ method $\pm$ S.E.M. Experiments were replicated in seven independent assays. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparisons were made at each time point between medium alone and treatment concentration. *, represents $P \leq 0.05$.

Evaluation of transforming growth factor β content in bovine seminal plasma

The content of TGF β in seminal plasma was evaluated in 33 individual bulls (Supplementary Fig. 2). Total TGF β -1 and TGF β -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 TGF β -1 and TGF β -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 $0.37 \pm 0.12\%$ of TGF β -1 and an average of $4.15 \pm 1.62\%$ of TGF β -2 was present in seminal plasma in the bioactive form. Using ejaculate volume and the concentration of total TGF β , we calculated the absolute content of TGF β -1 (63.4 ± 15.6 ng) and TGF β -2 (58.2 ± 11.2 ng) in individual ejaculates.

The effect of transforming growth factor β on BEND cell function

The effect of rhTGF β -1 (1, 10, or 100 ng/mL), or rhTGF β -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, rhTGF β -1 or rhTGF β -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 rhTGF β -1, rhTGF β -2 or medium alone (Fig. 5). In the absence of estradiol, expression of *TNF* (Fig. 5D and H) was

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 $2^{-\Delta Ct}$ method $\pm$ 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 $P \leq 0.05$ compared to medium alone.

increased following exposure to rhTGF β -1 or rhTGF β -2 compared to controls ($P \leq 0.05$), 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 rhTGF β -1 or rhTGF β -2 compared to controls ($P \leq 0.05$), but not

when estradiol was absent. Exposure of BEND cells to rhTGF β -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 ($P \leq 0.05$) following exposure to rhTGF β -2 in the presence of 0.1 nM estradiol (Fig. 5E and F).

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 rhTGF β . BEND cells were then exposed to either rhTGF β -1 (0, 1, 10 or 100 ng/mL; A, B, C, D), or rhTGF β -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 $2^{-\Delta Ct}$ method $\pm$ 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 $P \leq 0.05$ compared to appropriate control within an estradiol concentration.

Figure 6 Acute effects of transforming growth factor β on BEND cell gene expression. Expression of *CSF2* (A and E), *IL1B* (B and F), *IL6* (C and G) and *TNF* (D and H) in response to rhTGF β -1 (A, B, C and D) or rhTGF β -2 (E, F, G and H). BEND cells were treated with either medium alone, 100 ng/mL rhTGF β -1, or 10 ng/mL rhTGF β -2 for 0, 1, 3, 6 or 12 h. Culture medium was supplemented with 0.1 nM estradiol for 24 h prior to and during treatments. Data are presented as the mean relative expression normalized to *GAPDH* using the $2^{-\Delta C_t}$ method $\pm$ 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 at each time point between medium alone and treatment. *, represents $P \leq 0.05$.

The acute response of BEND cells to rhTGF β -1 (100 ng/mL), or rhTGF β -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 rhTGF β -1 or rhTGF β -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 rhTGF β -1 or rhTGF β -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 TGF β modulates BEND cell gene expression, seminal plasma was pre-incubated with TGF β neutralizing antibody (α TGF β) 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 μ g/mL of α TGF β 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 α TGF β (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 TGF β 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), rhTGF β -1 (1, 10 or 100 ng/mL), rhTGF β -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 rhTGF β -1 or rhTGF β -2 had no effect on either endometrial stromal or epithelial cells.

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-TGF β neutralizing antibody. Treatments were pre-incubated with 1, 5, 10, or 20 $\mu\text{g/mL}$ 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 $2^{-\Delta\text{Ct}}$ method $\pm$ 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 $P \leq 0.05$.

Effect of intrauterine infusion of semen components on endometrial gene expression

Endometrial response to saline, washed sperm, seminal plasma or rhTGF β -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 TGF β 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 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), rhTGF β -1 (1, 10 or 100 ng/mL), rhTGF β -2 (0.1, 1 or 10 ng/mL) or medium alone. Data are presented as the mean relative expression normalized to *GAPDH* using the $2^{-\Delta\text{Ct}}$ method $\pm$ 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 $P \leq 0.05$ compared to medium alone.

Figure 9 Effect of intrauterine infusion of semen components on endometrial gene expression. Expression of *CSF2* (A), *IL1B* (B), *IL6* (C) and *TNF* (D) was evaluated in endometrial tissue collected by cytobrush 12 h after intrauterine infusion with 0.5 mL of either saline, washed sperm, seminal plasma, or 10 ng of rhTGFβ-1. Each treatment was infused into five individual cows at the time of behavioral estrus. Data are presented as the mean relative expression normalized to *GAPDH* using the $2^{-\Delta Ct}$ method $\pm$ S.E.M. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparisons were made between each treatment.

the role of semen components in modulating the post-coital 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 semen 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 embryokine 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 TGF β -1 and TGF β -2 isoforms and elicits endometrial inflammation similar to that induced by seminal plasma exposure (Robertson 2005). TGF β -1, TGF β -2 and TGF β -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 TGF β -1 and TGF β -2 in both active and latent forms. Following insemination in mice, 70% of TGF β in uterine fluid is present in the active form, suggesting that activation of TGF β 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 TGF β 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 TGF β comparisons to other species can be confounded by ejaculate volume and site of semen deposition. Here we report the absolute content of bull semen TGF β -1 and TGF β -2 as 63 ng and 58 ng, respectively. Comparatively, human seminal plasma contains 220–240 ng/mL and 5–18 ng/mL of TGF β -1 and TGF β -2 (Nocera & Chu 1995), while mouse and swine semen TGF β -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 TGF β , by use of a pan-specific antibody, mediated a moderate reduction in *IL6* gene expression, future experiments should clarify this response using inhibitors of TGF β 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 TGF β 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 rhTGF β -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 TGF β -1 or TGF β -2 increased BEND cell *IL6* expression in an estradiol-dependent manner. Conversely, neither TGF β -1 nor TGF β -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 TGF β 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>.

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

J J B conceived the study. J A R and J J B wrote the paper and analyzed data. J A R, L A I, B R H, P C C M and R L P performed experiments.

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