

Research Article

Seminal plasma modulates expression of endometrial inflammatory mediators in the bovine[†]

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Abstract

Seminal plasma has conventionally been viewed as a transport and survival medium for mammalian sperm; however, its role now extends beyond this process to actively targeting female tissues. Studies in rodents, swine, and humans demonstrate that seminal plasma induces molecular and cellular changes within the endometrium or cervix following insemination. Seminal-plasma-induced alterations to the maternal environment have been theorized to facilitate embryo development, modulate maternal immunity toward the conceptus, and potentially improve pregnancy success. It is unknown if bovine seminal plasma modulates the uterine environment following insemination in the cow, where routine use of artificial insemination reduces maternal exposure to seminal plasma. We hypothesize that seminal plasma modulates the expression of inflammatory mediators in the endometrium, altering the maternal environment of early pregnancy. In vitro, seminal plasma altered intact endometrial explant expression of *CSF2*, *IL1B*, *IL6*, *IL17A*, *TGFB1*, *IFNE*, *PTGS2*, and *AKR1C4*. Furthermore, endometrial epithelial cell *CSF2*, *CXCL8*, *TGFB1*, *PTGS2*, and *AKR1C4* expression were increased after seminal plasma exposure, while endometrial stromal cell *CSF2*, *IL1B*, *IL6*, *CXCL8*, *IL17A*, *TGFB1*, *PTGS2*, and *AKR1C4* expression were increased following seminal plasma exposure. Endometrial expression of *IL1B* was increased in the cow 24 h after uterine infusion of seminal plasma, while other evaluated inflammatory mediators remained unchanged. These data indicate that seminal plasma may induce changes in the bovine endometrium in a temporal manner. Understanding the role of seminal plasma in modulating the maternal environment may aid in improving pregnancy success in cattle.

Summary Sentence

Exposure of endometrial cells to seminal plasma alters gene expression of inflammatory mediators, reminiscent of the postcoital inflammatory response in other species.

Key words: seminal plasma, endometrium, endometrial epithelium, endometrial stroma, inflammation, insemination.

Introduction

Seminal plasma (SP) is conventionally thought to have a single purpose, a survival medium for spermatozoa during carriage to the oocyte. However, the role of SP is now recognized to extend beyond this process to also optimize reproductive outcomes by targeting the female reproductive tract [1]. The periconceptual and embryo implantation phase of early pregnancy is a vulnerable period of the reproductive process [2, 3]. In livestock species, disruptions to the maternal environment and capacity to support conceptus development are implicated in constraining fertility [4, 5]. The cellular and molecular environment of the uterus during the peri-implantation period of early pregnancy is critical for implantation success and optimal fetal and placental development. In most mammalian species, it has been proven that SP is not required to achieve pregnancy, which is demonstrated by the success of artificial insemination (AI), embryo transfer, and cloning. Indeed, studies have demonstrated that in cattle AI and embryo transfer pregnancy rates are equal to or greater than natural conception [6, 7]. Despite the fact that SP is not required for successful pregnancy, several studies in multiple species indicate that pregnancy outcomes are altered if females are not exposed to SP at conception. For example, in mice, surgical removal of the seminal vesicle glands of stud males causes a reduction in subsequent pregnancy rates, due to decreased embryo implantation [1]. In dairy and beef cattle, infusion of SP at the time of AI increases pregnancy rates by 4.6% and 6.7%, respectively, albeit not significantly [8]. However, surgical removal of the seminal vesicle gland in the bull maintains a typical 69% conception rate [9]. Studies in rodents, swine, equine, and humans show that SP induces numerous changes within the endometrium or cervix [10–19]. Studies in various species have described SP-induced changes of endometrial *CSF2*, *IL6*, *CXCL8*, *IL17A*, and *PTGS2* [10–19]. These changes are proposed to occur by activating a cascade of cytokine and leukocyte-mediated events induced by active molecules present in SP. Epithelial cytokines induced by SP exposure exert embryotrophic actions on the developing pre-implantation embryo and modulate maternal immunity toward the conceptus [20–23]. In the human and rodent, SP-derived transforming growth factor beta ($TGF\beta$) has been demonstrated to be one of the active molecules within the ejaculate to facilitate these maternal tract changes [24, 25]. Herein, we hypothesize that exposure of bovine endometrial tissue to SP will modulate the expression of inflammatory mediators and alter the maternal environment of early pregnancy. We have employed *in vitro* and *in vivo* exposure of bovine endometrial tissue to semen components to better understand the impact of SP on modulating the environment of the maternal reproductive tract. We have measured targeted genes known to be regulated by SP in other species, in addition to novel factors involved in inflammation of the endometrium (*IFNE*) or regulation of ovarian function (*OXTR*). These studies are an important addition to our understanding of SP's role in modulating the female reproductive tract environment of early pregnancy, particularly in domestic species such as cattle where SP is diluted during AI, potentially removing any paternally derived effects on the maternal tissues.

Materials and methods

All reagents were acquired from Fisher Scientific (Waltham, MA) unless otherwise stated.

Animal use and care

All animals were housed at the University of Florida, North Florida Research and Extension Center (NEFREC). All procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Semen and seminal plasma collection

Whole semen was collected from Angus bulls during routine breeding soundness evaluation. Whole semen was collected by electroejaculation from healthy bulls, evaluated and only processed further if the sample was free of blood, urine, and any other visual anomalies. Parameters pertaining to the breeding soundness evaluation were recorded for each individual sample; including volume, sperm motility, scrotal circumference, collection date, and bull ID. Collected semen volume ranged from 7.5 to 15 ml, gross motility ranged from 10% to 90%, and scrotal circumference ranged from 31 to 49 cm. Following collection, whole semen was placed on ice and transported to the laboratory for processing under aseptic conditions. A 200 μ l aliquot of whole semen was placed into a sterile microcentrifuge tube and stored at -20°C . The remaining ejaculate was centrifuged at $1000 \times g$ for 10 min at room temperature (RT) to facilitate collection of cell free SP and the ejaculate cell pellet. Cell-free SP was transferred to new labeled tubes in aliquots of 500–1000 μ l and stored at -20°C until use. The remaining cell pellet was stored at -20°C .

Preparations of pooled semen, seminal plasma, and cell pellet

A whole semen pool was prepared under aseptic conditions by combining 100 μ l whole semen from 11 individual bulls. Pooled whole semen was stored in 500 μ l aliquots at -20°C until use. The cell pellet from individual bulls was resuspended in sterile phosphate-buffered saline (PBS) to the same volume of the original ejaculate (achieving the original ejaculates sperm concentration). The volume of cell suspension from each bull corresponding to 10^8 total cells was pooled and stored in 500 μ l aliquots at -20°C . Ejaculates that contained a total volume of 5 ml or higher were used to generate the SP pool. The SP pool was prepared from the ejaculates of 21 individual bulls. Seminal plasma was pooled under aseptic condition by combining 1 or 2 ml of SP from each bull, and stored in 500 μ l aliquots at -20°C . Each SP aliquot was used a maximum of twice before being discarded to minimize any negative effects of repeated freeze/thawing.

Preparation of female reproductive tracts for endometrial dissection

Uteri from postpubertal, nonpregnant cattle with no gross evidence of genital disease or microbial infection were collected at the local slaughterhouse (breed was not defined). Whole reproductive tracts were transported to the laboratory within approximately 3 h for further processing at RT. Reproductive tracts from a total of 36 animals were used for all experiments. The stage of the reproductive cycle (stage of estrous cycle) was determined by examination of ovarian morphology and vasculature of the corpus luteum (CL), according to Ireland et al. [26], and only reproductive tracts between days 1 and 10 of the estrous cycle were used herein. Reproductive tracts were prepared as previously described for both intact explants and

semi-purified epithelial and stromal cell isolation [27]. Briefly, individual uteri were freed of surrounding fat and connective tissue and rinsed with 70% ethanol. An incision along the major curvature of the uterine horn was made to expose the endometrium ipsilateral to the CL. The exposed endometrium was washed twice with Dulbecco phosphate-buffered saline containing 50 IU/ml of penicillin, 50 μ g/ml of streptomycin, and 2.5 μ g/ml amphotericin B to remove mucus and potential microorganisms. Tissues were then immediately processed for isolation of intact endometrial explants or semi-purified epithelial or stromal cells (below).

Preparation of whole endometrial explants and in vitro culture

Endometrial explants were obtained from intracaruncle tissue using an 8-mm diameter biopsy punch (Stiefel Laboratories Ltd, Research Triangle Park, NC) and cut away from the underlying myometrium. Endometrial biopsies were immediately washed twice with Hank balanced salt solution (HBSS). A single biopsy was placed into a well of a 6-well culture plate (TPP, Trasadingen, Switzerland) containing 3 ml of complete culture medium (RPMI 1640 medium, 10% FBS, 2 mM of L-Glutamine, 50 IU/ml of penicillin, 50 μ g/ml of streptomycin, and 2.5 μ g/ml amphotericin B) with the specified treatment (below). Each experimental replicate is derived from explants of individual cows. Six independent experiments were performed for each treatment, represented by explants from six individual cows.

Isolation and culture of bovine endometrial epithelial and stromal cells

Endometrial tissue was dissected and processed as described previously by Turner et al. [28]. Briefly, thin strips of endometrium were dissected away from the underlying serosa and placed directly into HBBS containing 50 IU/ml of penicillin, 50 μ g/ml of streptomycin, and 2.5 μ g/ml amphotericin B. Tissue pieces were transferred into a centrifuge tube containing HBSS and placed into a water bath at 37°C. After 10 min, the sterile digestive solution (HBSS containing 100 mg BSA, 125 CDU/mg collagenase II, 250 BAEE trypsin and 4% DNase I) was added to the endometrial tissue and digested for 1 h in a shaking water bath at 37°C. The cell suspension was then filtered through a 40- μ m mesh and resuspended in HBSS containing 10% FBS. The suspension was centrifuged at 700 \times g for 7 min at RT and resuspended in warm complete culture medium (as above). Cells were cultured in 30 ml of equilibrated complete culture medium in 75 cm² flasks (Greiner Bio-one, Monroe, NC) at 39°C in a humidified atmosphere of air with 5% CO₂. After 18 h of culture, unattached epithelial cells in suspension were transferred to a new flask leaving semi-purified stromal cells attached. Both epithelial and stromal cells were cultured for an additional 48 h before plating at a final concentration of 1.5 \times 10⁵ cells/ml in 24-well culture plates (TPP, Trasadingen, Switzerland) at a final volume of 500 μ l in complete culture medium. Cells were equilibrated for 24 h before treatment (below).

Cell culture challenge of endometrial explants or endometrial cells with semen components

Intact endometrial explants or endometrial cells were exposed to either complete culture medium alone or medium containing 1%, 2%, 5%, 10%, or 20% v/v SP; 5% v/v whole semen (SM); or 5% v/v cell pellet (CP). Treatments were added to complete culture medium according to experimental design and equilibrated at 37°C prior to

exposure of explants or cells. A minimum of six replicates were performed for each treatment or time point, with each replicate representative of endometrial material from an individual cow. Following application of treatment, explants or endometrial cells were incubated at 39°C in a humidified 5% CO₂ environment. Initial dose experiments using endometrial explants or cells were performed for a total of 24 h. Time course experiments of both explants and endometrial cells utilized complete culture medium alone or medium containing 5% v/v SP for 0, 15, 30, 60, 90, and 120 min. Following treatment, explants were weighed prior to bisecting and storage at -20°C in Trizol for RNA extraction. Epithelial and stromal cells cultures were stored at -20°C in Qiagen RLT lysis buffer (Qiagen, Hilden, Germany).

Synchronization and intrauterine infusion of cows

A total of 54 multiparous nonlactating Angus beef cows were randomly assigned to receive 1 ml of treatment: (1) saline, (2) conventional semen from a single sire, (3) pooled SP alone, or (4) a combination of conventional semen and SP. Each treatment was preloaded in 0.5 ml semen straws under aseptic conditions. Saline and SP were filter sterilized using a 0.4- μ m syringe filter before loading into straws.

Estrous synchronization was performed in all cows by administering GnRH (Factrel; 100 μ g gonadorelin hydrochloride; Zoetis Animal Health, Parsippany, NJ), followed by PGF_{2 α} treatment (Lutalyse; 25 mg dinoprost tromethamine; Zoetis Animal Health) 7 days later. Transrectal ultrasound (Ibex ultrasound with 5 MHz multifrequency transducer: E. I. Medical Imaging, Loveland, CO) was performed to confirm the presence and location of a dominant/ovulatory follicle at the time of PGF_{2 α} treatment and again at the time of intrauterine infusion. Treatments were administered into the uterine body 48 h after PGF_{2 α} treatment by standard AI technique. Endometrial biopsies were collected from both uterine horns, ipsilateral and contralateral to the ovulatory follicle. Briefly, animals received local anesthesia by administering 4 ml of lidocaine to the epidural space between the second and third coccygeal vertebrae. Endometrial biopsy forceps (Jorgensen Laboratories, Loveland, CO) were passed into the uterine horn, guided by rectal palpation. Once in place, the endometrium was gently pressed into the jaw of the biopsy forceps by rectal palpation and the endometrium clipped off by closing the instrument jaw. The process was repeated to facilitate the collection of endometrial tissue from both uterine horns, ipsilateral and contralateral to the dominant follicle. Endometrial biopsies were rinsed in sterile PBS, bisected into two pieces, and stored in RNAlater (Invitrogen) until RNA extraction or fixed in 10% neutral-buffered formalin. Biopsies from 43 cows with a visible dominant follicle were used for further analysis, and 11 cows were excluded from analysis as no visible dominant follicle was observed, indicating that they either ovulated prior to administration of treatments or failed to respond to synchronization of estrus.

Extraction and purification of RNA from endometrial tissues or cells

RNA was extracted from endometrial explants and biopsies using Trizol. Briefly, endometrial tissue was homogenized using a plastic pellet pestle (Kimble, Rockwood, TN). Homogenates were centrifuged at 1200 \times g for 5 min at 4°C and supernatants were transferred to clean centrifuge tube. A total of 200 μ l of chloroform was added to each homogenate and incubated at RT for 5 min. Samples were centrifuged at 1200 \times g for 15 min at 4°C. The RNA-rich

aqueous supernatant was transferred to a new tube and 500 μ l of 100% isopropanol was added to the sample and incubated at RT for 10 min. Samples were centrifuged at $1200 \times g$ for 15 min at 4°C and supernatant was removed leaving the RNA pellet. The pellet was washed with 1 ml of 75% ethanol and centrifuged at $1200 \times g$ for 10 min at 4°C. The RNA pellet was resuspended in 50 μ l of RNAase-free water. Total RNA was extracted from semi-purified endometrial cells using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen). Extracted RNA was quantified using a NanoDrop ND1000 spectrophotometer. A total of 1 μ g of RNA was subjected to reverse transcription using the Verso cDNA synthesis kit according to the manufacturer's instruction, including a genomic DNA wipe-out procedure. The recommended thermal cycling conditions for reverse transcription were one cycle of cDNA synthesis at 42°C for 30 min followed by inactivation at 95°C for 2 min.

Quantitative real-time RT-PCR

Primers were designed using the NCBI database and initial specificity verified by BLAST to ensure no cross-reactivity with other loci (Supplemental Table S1). Amplification efficiency was evaluated for each primer by performing serial dilutions of cDNA. All primers met MIQE guidelines for further use (Pearson correlation coefficient $r > 0.98$ and efficiency between 90% and 110%). Quantitative real-time RT-PCR was performed in 20 μ l reactions using iTaq Universal SYBR green chemistry (Bio-Rad, Hercules, CA) and 100 nM of each forward and reverse primer. A Bio-Rad CFX Connect light cycler was employed to perform quantitative PCR (Bio-Rad) using a two-step protocol and the recommended thermal cycling conditions outlined below: 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. A three-step protocol was employed for *IL17A* and *OXTR* primers with an annealing temperature of 63°C. 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 nonspecific amplification. PCR products were electrophoresed on a 1% agarose gel containing Diamond Dye (Promega, Madison, WI) and visualized under UV illumination to confirm predicted PCR product size. All cell culture data were normalized independently to *ACTB* mRNA expression, while in vivo expression was normalized to the arithmetic mean of *ACTB* and *GAPDH* mRNA expression using the $\Delta\Delta C_t$ method.

Histology and immunohistochemistry of endometrial tissue

Paraffin-embedded sections were cut at 7 μ m and stained using hematoxylin and eosin. Immunohistochemistry was performed to detect IL-1 β (#MBS2026412; MyBioSource; San Diego, CA) using Alexafluor 488 secondary detection (Invitrogen, Carlsbad, CA) and DAPI counterstaining (Vector Labs, Burlingame, CA). Tissue sections underwent antigen retrieval using Sodium Citrate buffer (pH 6.0) in a pressure cooker for 3 min. Images were taken using a Zeiss Axioplan microscope and Axiovision software (Zeiss, Gottingen, Germany).

Statistical analysis

SPSS software V24.0 (IBM Analytics, Armonk, NY) was used for statistical analysis. Relative gene expression data was log transformed and analyzed using the generalized linear mixed model with pairwise comparisons. Treatment, dose, time, stage of estrous cycle, and uterine horn were used as fixed factors and replicate was included as a

random variable. Interactions were assessed as appropriate in either dose experiments or time course experiments. Pairwise comparisons were made between individual time points or concentrations with vehicle treated controls. The stage of estrous cycle had no effect on responsiveness and was removed from the analysis. Data for individual replicates according to the stage of estrous cycle are presented in Supplemental Figure S1. Data are presented as mean + standard error of the mean. A *P* value of ≤ 0.05 was assumed statistically significant.

Results

The effect of semen components on ex vivo endometrial explant gene expression

To assess the capacity of semen components to modulate expression of inflammatory mediators, intact endometrial explants were exposed to either vehicle control medium alone, 2%, 5%, 10%, or 20% v/v SP, 5% v/v semen cell pellet (CP), or 5% v/v whole semen (SM) for 24 h (Figure 1).

Expression of endometrial *CSF2*, *IFNE*, *PTGS2*, and *AKR1C4* were increased following exposure to SP compared to vehicle treated controls, whereas expression of *IL6*, *IL17A*, and *TGFB1* were down-regulated following exposure to SP. Interestingly, explant exposure to 5% SM only modulated *IL6*, *AKR1C4*, and *OXTR* expression compared to vehicle treated controls. Exposure to 5% CP had no effect on any genes measured.

Explant exposure to 10% SP maximally increased expression of *CSF2* by 29.2-fold compared to vehicle treated explants (Figure 1A; $P < 0.05$). Exposure to 20% SP increased *PTGS2* and *AKR1C4* expression maximally by 5.16- and 15.0-fold compared to controls, respectively (Figure 1G and H; $P < 0.05$). Treatment of explants with 20% SP reduced *IL6*, *IL17A*, and *TGFB1* expression by 85.7%, 89.4%, and 97.3% , respectively, compared to vehicle treated control expression, (Figure 1B, D, E; $P < 0.05$). On the other hand, expression of *IFNE* was increased by 1.7-fold following exposure to 20% SP compared to control treated explants (Figure 1F; $P < 0.05$). Explant expression of *CXCL8* or *IL1B* was not modulated by any semen components (Figure 1C). The expression of *IFNG* was not detectable in all in vitro experiments (data not shown).

Explant viability was assessed by measuring LDH activity on supernatants after culture (Supplemental Figure S2). Minimal increases in LDH activity were observed following treatment, with the exception of a 5.6-fold increase in the 20% SP treatment group compared to uncultured medium alone. The proportion of LDH activity following treatment with 20% SP was comparable to treatment with medium containing 1% Triton X-100.

Acute effects of semen components on ex vivo endometrial gene expression

Explants were exposed to 5% SP or vehicle control medium alone for 0, 15, 30, 60, 90, and 120 min to assess acute effects of SP on gene expression (Figure 2). Treatment with SP increased *CSF2*, *TGFB1*, *IFNE*, *PTGS2*, and *IL1B* expression during the experimental period (Figure 2A, D, E, F, G, J; $P < 0.05$), while SP treatment reduced overall expression of *IL6* and *CXCL8* (Figure 2B and C; $P < 0.05$). An effect of culture time and a treatment by time interaction was observed for *IFNE* expression (Figure 2F; $P < 0.05$), while an effect of culture time was observed for *PTGS2* expression (Figure 2E; $P < 0.05$). Specifically, treatment with SP increased *CSF2*, *TGFB1*, *IFNE*, and *PTGS2* expression compared to vehicle treated controls at 120 min (Figure 1A, E, F, G; $P < 0.05$). Interestingly, expression

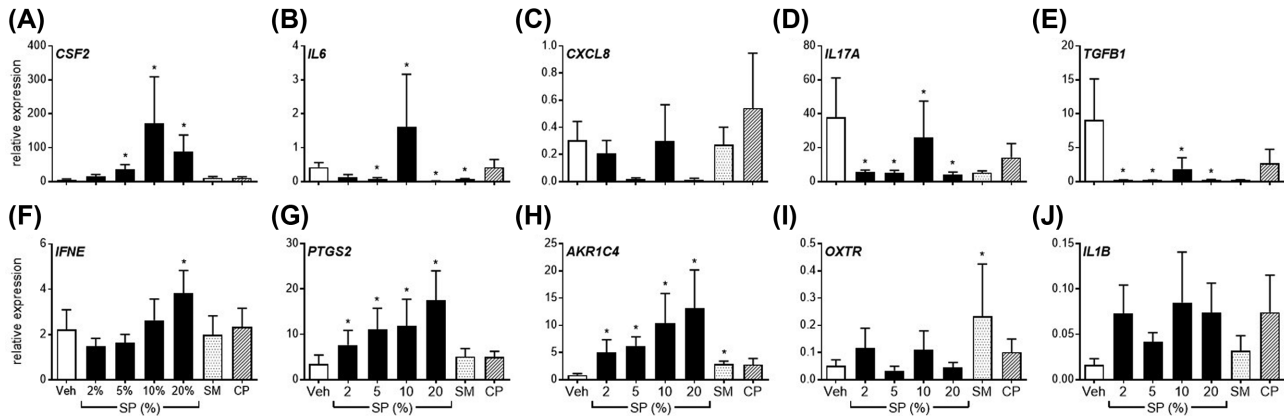


Figure 1. Effect of semen components on endometrial explant gene expression. Intact endometrial explant expression of *CSF2*, *IL6*, *CXCL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS2*, *AKR1C4*, *OXTR*, and *IL1B* in response to seminal plasma (SP; 2%, 5%, 10%, 20%), 5% whole semen (SM), 5% semen cell pellet (CP), or vehicle medium alone (Veh) after 24 h. Data are presented as the mean relative expression + SEM, from six independent experiments using explants from six individual cows. Data were log transformed and analyzed using the generalized linear mixed model with pairwise comparisons only made with vehicle treated control. *, *P* value of ≤ 0.05 .

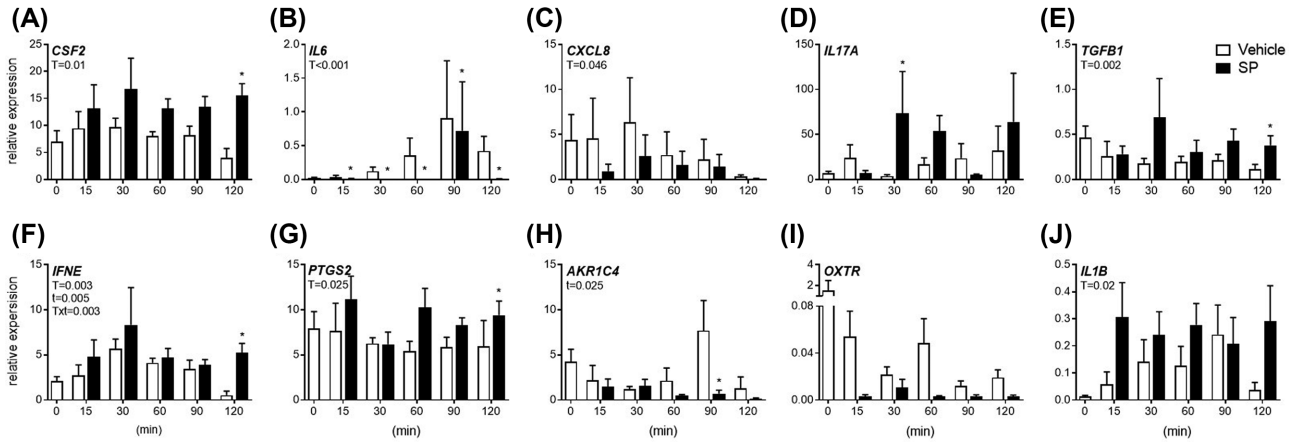


Figure 2. Acute effects of semen components on endometrial explant gene expression. Intact endometrial explant expression of *CSF2*, *IL6*, *CXCL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS2*, *AKR1C4*, *OXTR*, and *IL1B* in response to 5% seminal plasma (SP) or control medium alone (vehicle). Gene expression was measured in terminal experiments starting at 15 min, up to 120 min. Data are presented as the mean relative expression + SEM from six independent experiments. Data were log transformed and analyzed using the generalized linear mixed model with pairwise comparisons made with vehicle treated control within each time point. *, *P* value of ≤ 0.05 . T, treatment effect; t, time effect; Ttx, treatment by time interaction.

of *IL6* was reduced compared to vehicle treated controls at 15, 30, 60, 90, and 120 min (Figure 2B; $P < 0.05$). There was no effect of treatment on *OXTR* expression at any time point (Figure 2I).

The effect of semen components on semi-purified endometrial epithelial and stromal cell gene expression

Semi-purified endometrial epithelial (Figure 3) and stromal (Figure 4) cells were exposed to either vehicle control medium alone, 1%, 2%, or 5% v/v SP, 5% v/v semen cell pellet (CP), or 5% v/v whole semen (SM) for 24 h. Purity of epithelial and stromal cells was confirmed by microscopy and flow cytometry ($56 \pm 16\%$ and $95 \pm 3\%$, respectively; Supplemental Figure S3).

Exposure to 5% SM increased epithelial expression of *CSF2*, *CXCL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS2*, and *AKR1C4* compared

to vehicle treated controls (Figure 3A, C–H; $P < 0.05$). Interestingly, exposure of epithelial cells to 5% CP maximally increased expression of all evaluated genes compared to vehicle treated controls (Figure 3; $P < 0.05$), while treatment of epithelial cells with SP increased expression of *CSF2*, *CXCL8*, *TGFB1*, *PTGS2*, and *AKR1C4* (Figure 3A, C, E, G and H; $P < 0.05$). Specifically, exposure of epithelial cells to 2% SP increased *CSF2*, *CXCL8*, *TGFB1*, *PTGS2*, and *AKR1C4* expression by 9.8, 28.9, 69.3, 29.9, and 27.1-fold, respectively ($P < 0.05$). Epithelial cells did not modulate expression of *IL1B* to any semen component (Figure 3J).

Exposure to 5% SM increased stromal cell expression of *CSF2*, *IL17A*, *TGFB1*, *IFNE*, *PTGS2*, and *AKR1C4* compared to vehicle treated controls (Figure 4A, D–H; $P < 0.05$). Exposure of stromal cells to 5% CP increased expression of *TGFB1*, *PTGS2*, and *OXTR* compared to vehicle treated controls (Figure 4E, G, I; $P < 0.05$), while treatment with SP increased stromal cell expression of *CSF2*,

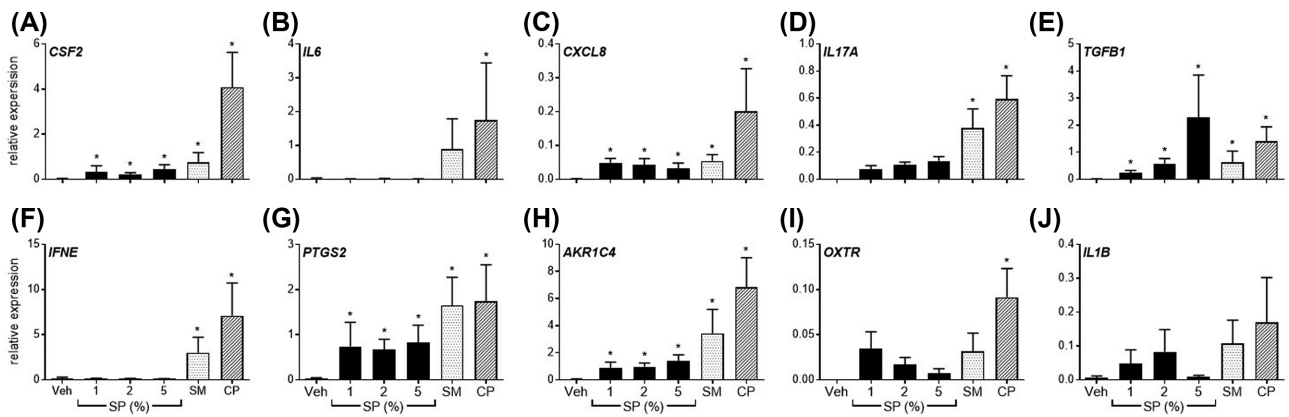


Figure 3. Effect of semen components to alter gene expression of semi-purified endometrial epithelial cells. Semi-purified endometrial epithelial cell expression of *CSF2*, *IL6*, *CXCL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS2*, *AKR1C4*, *OXTR*, and *IL1B* in response to seminal plasma (SP; 1%, 2%, 5%), 5% whole semen (SM), 5% semen cell pellet (CP), or vehicle medium alone (Veh) after 24 h. Data are presented as the mean relative expression + SEM from six independent experiments. Data were log transformed and analyzed using the generalized linear mixed model with pairwise comparisons made with vehicle treated control. *, P value of ≤ 0.05 .

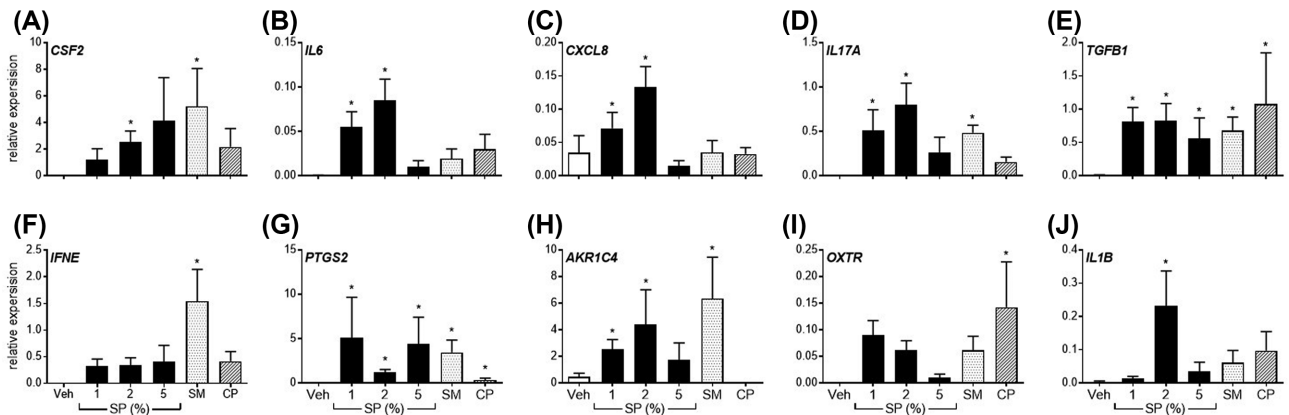


Figure 4. Effect of semen components to alter gene expression of semi-purified endometrial stromal cells. Semi-purified endometrial stromal cell expression of *CSF2*, *IL6*, *CXCL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS2*, *AKR1C4*, *OXTR*, and *IL1B* in response to seminal plasma (SP; 1%, 2%, 5%), 5% whole semen (SM), 5% semen cell pellet (CP), or vehicle medium alone (Veh) after 24 h. Data are presented as the mean relative expression + SEM from six independent experiments. Data were log transformed and analyzed using the generalized linear mixed model with pairwise comparisons made with vehicle treated control. *, P value of ≤ 0.05 .

IL6, *CXCL8*, *IL17A*, *TGFB1*, *PTGS2*, and *AKR1C4* compared to vehicle treated controls (Figure 4A–E, G, H; $P < 0.05$). Specifically, exposure of stromal cells to 2% SP increased *CSF2*, *IL6*, *CXCL8*, *TGFB1*, *PTGS2*, *AKR1C4*, and *IL1B* expression by 2248.0, 134.4, 2.1, 74.1, 165.4, 9.6, and 69.0-fold, respectively, compared to vehicle treated controls, respectively ($P < 0.05$).

Cell viability of semi-purified epithelial and stromal cells was assessed using the MTT assay following treatment for 24 h (Supplemental Figure S3D and H). Viability of epithelial cells was reduced by 76.5% of vehicle treated controls only after exposure to 20% SP. Stromal cell viability was reduced by 26.3%, 43.1%, and 35.7% of vehicle treated controls following treatment with 5%, 10%, and 20% SP, respectively.

Acute effects of semen components on semi-purified endometrial epithelial and stromal cells

Semi-purified endometrial epithelial (Figure 5) and stromal (Figure 6) cells were exposed to 5% SP or vehicle control medium alone for 0, 15, 30, 60, 90, and 120 min to assess acute effects of SP on gene expression.

Treatment of epithelial cells with SP increased *CSF2*, *IL6*, *IL17A*, *TGFB1*, *IFNE*, *PTGS2*, *AKR1C4*, and *IL1B* expression during the experimental period (Figure 5A, B, D–H, J; $P < 0.05$). Epithelial expression of *CXCL8* and *OXTR* were unaffected by treatment (Figure 5C and I). Specifically, epithelial expression of *CSF2*, *TGFB1*, *PTGS2*, and *AKR1C4* were elevated above vehicle treated controls starting at 15 min for the 120 min duration of the experiment ($P < 0.05$). There was no effect of culture time on expression of any epithelial genes assessed.

Treatment of stromal cells with SP increased expression of *CSF2*, *IL6*, *CXCL8*, *IL17A*, *TGFB1*, *PTGS2*, *AKR1C4*, and *OXTR* during the experimental period (Figure 6; $P < 0.05$). Expression of stromal cell *CSF2*, *IL6*, *TGFB1*, and *PTGS2* were all increased compared to the vehicle treated controls starting at 15 min for the 120 min duration of the experiment (Figure 6A, B, E, G; $P < 0.05$).

Effect of intrauterine infusion of seminal plasma on endometrial gene expression

Endometrial biopsies were collected 24 h after intrauterine infusion of semen, SP, or saline in synchronized cows to determine the

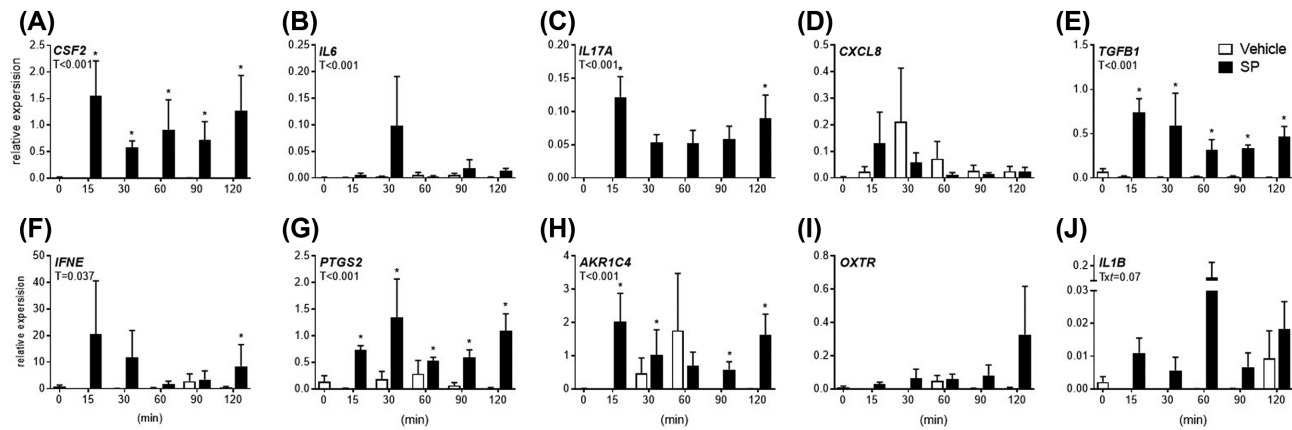


Figure 5. Acute effects of semen components on semi-purified endometrial epithelial cell gene expression. Semi-purified endometrial epithelial cell expression of *CSF2*, *IL6*, *CXCL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS2*, *AKR1C4*, *OXTR*, and *IL1B* in response to 5% seminal plasma (SP) or vehicle medium alone (vehicle). Gene expression was measured in terminal experiments starting at 15 min, up to 120 min. Data are presented as the mean relative expression + SEM from six independent experiments. Data were log transformed and analyzed using the generalized linear mixed model with pairwise comparisons made with vehicle treated control within each time point. *, *P* value of ≤ 0.05 . T, treatment effect.

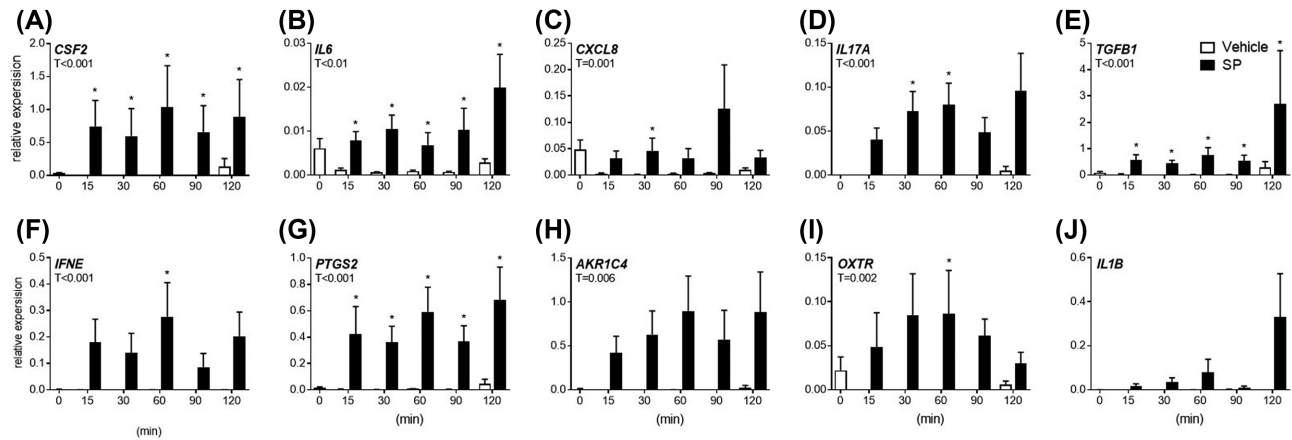


Figure 6. Acute effects of semen components on semi-purified endometrial stromal cell gene expression. Semi-purified endometrial stromal cell expression of *CSF2*, *IL6*, *CXCL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS2*, *AKR1C4*, *OXTR*, and *IL1B* in response to 5% seminal plasma (SP) or vehicle medium alone (vehicle). Gene expression was measured in terminal experiments starting at 15 min, up to 120 min. Data are presented as the mean relative expression + SEM from six independent experiments. Data were log transformed and analyzed using the generalized linear mixed model with pairwise comparisons made with vehicle treated control within each time point. *, *P* value of ≤ 0.05 . T, treatment effect.

endometrial expression of inflammatory mediators (Figure 7). Histological evaluation of endometrial biopsies demonstrated the presence of heterogeneous cell types, including luminal and glandular epithelium, stroma, vasculature, and immune cells (Figure 8A). Endometrial expression of *IL1B* was increased by 2.2-fold in the SP treatment group compared to the saline infused controls (Figure 7E; $P < 0.05$). The effect of SP-induced *IL1B* expression was due mainly to increased expression in the contralateral horn where expression was increased by 2.6-fold compared to the contralateral horn of the saline infused group. Immunohistochemistry of endometrial biopsies confirmed the presence of IL-1 β in endometrial tissue in all treatment groups (Figure 8B). Expression of IL-1 β was concentrated in the endometrial luminal epithelium, with some immunoreactivity present in glandular epithelium and less so in the stromal tissue. A treatment effect was also observed in the semen + SP group where *PTPRC* (formerly CD45) expression was reduced by 63.1%

(Figure 7I; $P < 0.05$). Reduced *PTPRC* expression was due primarily to a 62.0% reduction in the ipsilateral horn of the semen + SP infused group compared to the ipsilateral horn of the saline infused controls. Interestingly, *PTPRC* expression in the contralateral horn was elevated in the SP infused group compared to the semen + SP group (Figure 7I; $P < 0.05$), while expression of *PTGS2* in the ipsilateral horn of saline treated cows was 4.6-fold higher than expression of the ipsilateral horn in SP treated cows (Figure 7G; $P < 0.05$). Expression of endometrial *CSF2* in the contralateral horn was increased by 3.9-fold following semen infusion compared to the contralateral horn of saline infused cows (Figure 7A; $P < 0.05$), while saline infused cows had a 3.8-fold increase in *CSF2* expression of the ipsilateral horn compared to the contralateral horn (Figure 7A; $P < 0.05$). Expression of *IL17A*, *AKR1C4*, and *TGFB1* were increased in the ipsilateral horn of the semen + SP group (Figure 7J-L; $P < 0.05$), while *AKR1C4* expression was increased in the ipsilateral horn

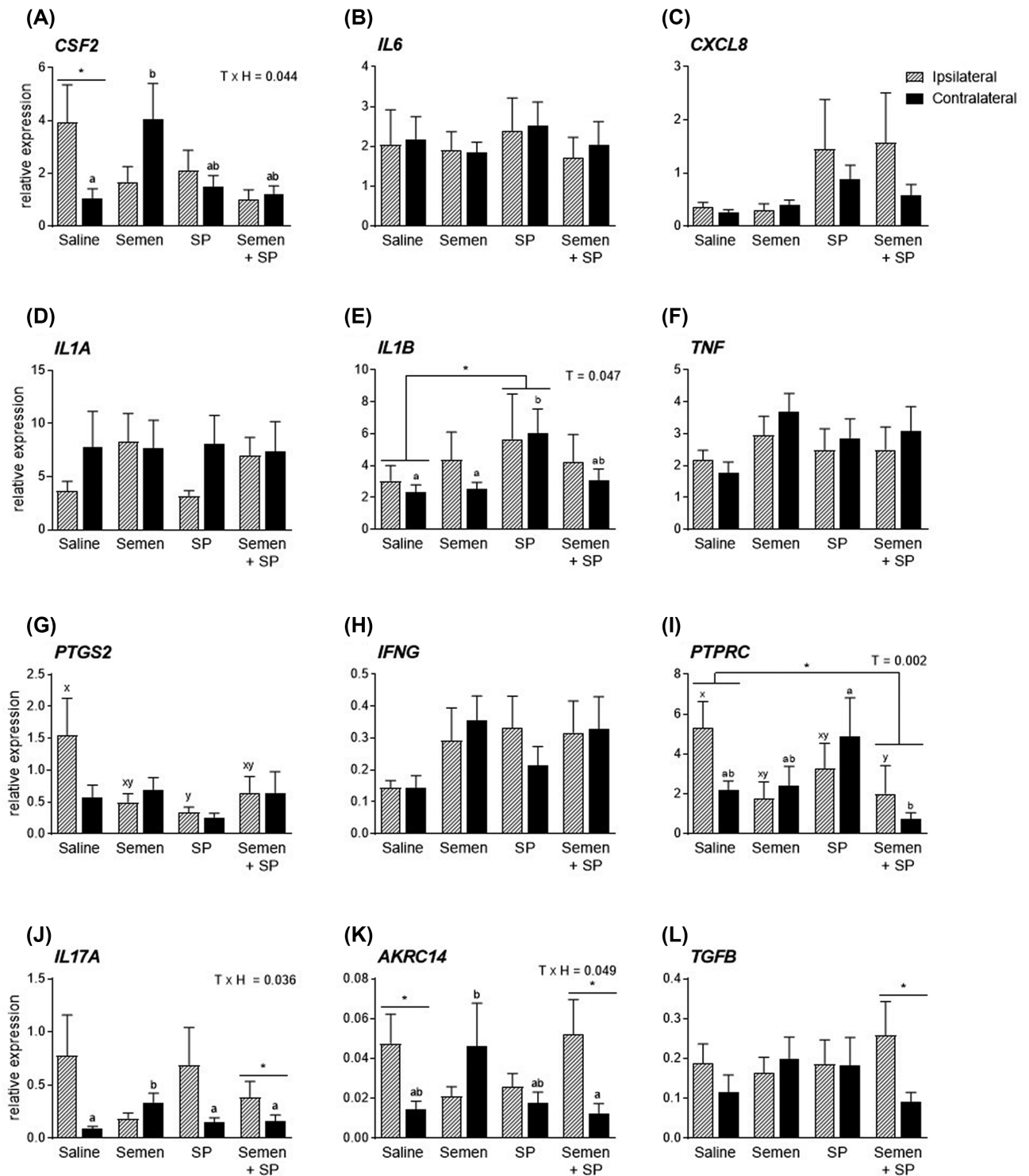


Figure 7. Effect of intrauterine infusion of semen on endometrial gene expression. Endometrial biopsies were collected 24 h following intrauterine infusion of conventional semen ($n = 12$), pooled seminal plasma (SP; $n = 11$), a combination of conventional semen and SP ($n = 9$), or saline ($n = 11$) as a control. Biopsies were collected from uterine horns ipsilateral (hatched bars) and contralateral (solid bars) of the dominant follicle, identified by ultrasound the day before intrauterine infusion. Expression of *CSF2*, *IL6*, *CXCL8*, *IL1A*, *IL1B*, *TNF*, *PTGS2*, *IFNG*, and *PTPRC* were evaluated by real-time RT-PCR. Data are presented as the mean relative expression + SEM. Data were analyzed using repeat measures in the generalized linear mixed model with pairwise comparisons. The dam was used as the subject, treatment, horn, and treatment \times horn were used as fixed effect. *, P value of ≤ 0.05 . Superscripts a and b refer to differences observed between treatments within the contralateral horn; superscripts x and y refer to differences observed between treatments within the ipsilateral horn. T, treatment effect; TxH, treatment by horn interaction.

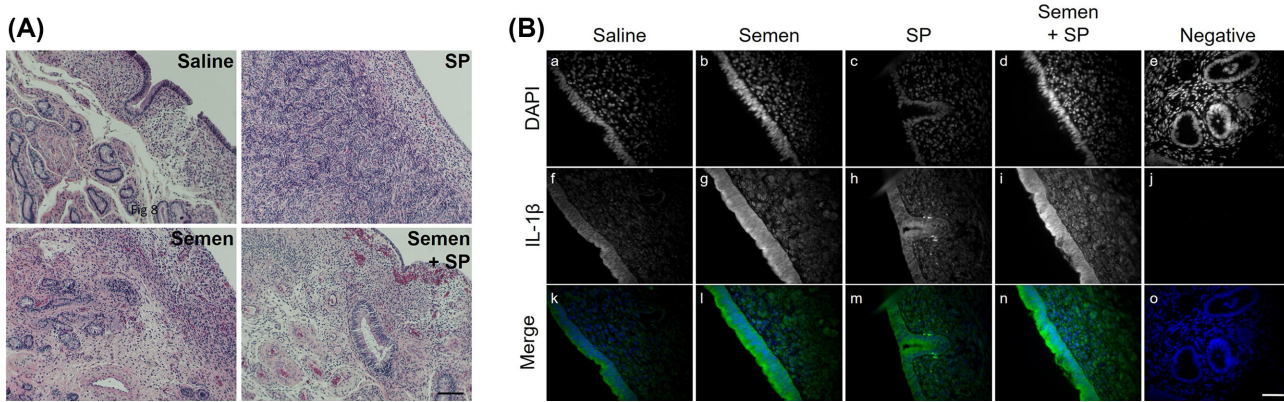


Figure 8. Effect of intrauterine infusion of semen components on endometrial expression of IL-1 β . Representative micrographs of endometrial biopsies collected 24 h following intrauterine infusion of saline, conventional semen, pooled seminal plasma (SP), or a combination of conventional semen and SP. (A) Biopsies were fixed in neural-buffered formalin, paraffin-embedded, sectioned, and H&E stained. Scale bar represents 100 μ m. (B) Biopsies were fixed in neural-buffered formalin, paraffin embedded, sectioned, and stained for immunoreactive IL-1 β . Separate black and white images are shown for DAPI (a–e) and IL-1 β staining (f–j), in conjunction with a merged image showing DAPI in blue and IL-1 β in green (k–o). A negative control (e, i, o) is shown that was not incubated with primary antibody. Scale bar represents 50 μ m.

compared to the contralateral horn of saline infused cows (Figure 7K; $P < 0.05$).

Seven days following uterine infusion, serum progesterone was evaluated and no differences were observed between the treatment groups (Supplemental Figure S4B).

Discussion

The cell-free component of semen, SP, has been demonstrated to modulate the molecular and cellular environment of the maternal reproductive tract of rodents, swine, horse, and humans [18–20, 29, 30]. The data herein describe the impact of semen, and SP specifically, in modulating the endometrial environment of the bovine. Our data suggest that while SP alters the expression of endometrial inflammatory genes in isolated culture systems, the degree of alteration in the cow endometrium 24 h after uterine infusion is significantly less.

Pregnancy in many species, including cattle can be readily achieved in the absence of SP, using IVF or AI using washed sperm. Over 10 million bovine AIs are performed each year in the USA, where SP is considerably diluted during semen extension to maximize the efficiency of a single ejaculate [31]. Studies have demonstrated that pregnancy rates from cows undergoing AI or embryo transfer in the absence of SP are at least equal to natural conception [6, 7]. However, data in the rodent suggest that SP has the capacity to modulate the maternal environment and improve pregnancy outcomes, including offspring phenotype [1]. Pregnancies of mice achieved in the absence of SP result in increased embryo mortality and metabolic perturbations in resultant offspring due to a compromised uterine/oviductal environment [1]. In the bovine the addition of SP at the time of AI increases conception rates, albeit not significantly, suggesting a potential role of SP in cattle reproduction that requires further investigation [8]. Indeed, similar evidence exists in the swine and human, where SP exposure at the time of AI or embryo transfer increases conceptions rates [30, 32].

The current study indicates that whole endometrial explants increase expression of *CSF2*, *PTGS2*, and *AKR1C4* following exposure to SP, but not semen cells. This is consistent with SP-induced

modulation reported in humans, rodents, and swine. However, whole endometrial explants are complex tissues comprised of epithelial, stromal, endothelial, and immune cells which may mediate various responses to semen components. Thus, we assessed the capacity of semi-purified endometrial epithelial or stromal cells to respond to SP. Purification of epithelial and stromal cells in our model system was consistent with the purity achieved by others, with epithelial purity difficult to achieve above 70% purity [28]. In semi-purified cultures, the responses to SP were more pronounced than in the complex explant cultures. Both epithelial and stromal cells responded to whole semen, which is consistent with cells responding to SP alone. Interestingly, there was a greater capacity of epithelial cells to respond to semen cell pellet, compared to stromal cells. This may be a reflection of the anatomical separation of the stromal cell compartment from the luminal space where semen (and SP) may be present following insemination. Indeed, the higher degree of responsiveness of semi-purified epithelial cells compared to endometrial explants may due to the lower proportional density of epithelial cells present in explants compared to stromal and other cell types which make up the majority of explant biopsies (as described in the histological evaluation).

Previous work in rodents has demonstrated that SP exposure at insemination improves pregnancy outcomes and reduces the incidence of metabolic syndrome in offspring [1]. It is hypothesized that the mechanism of SP improving pregnancy outcomes is multifaceted, improving expression of maternal embryokines to support preimplantation development, facilitating tissue remodeling for implantation and placental development, and finally modulation of the maternal immune response to the semi-allogeneic conceptus [23]. Data here from *in vitro* experiments support the hypothesis that SP may aid in the expression the embryokine *CSF2*, which is known to be beneficial to preimplantation embryo development in the cow, rodent, and human [33–36]; however, it is important to note that SP did not alter the expression of endometrial *CSF2* 24 h following intrauterine infusion.

The postcoital inflammatory response of rodents reported by Yanagimich in 1963 [19] was later demonstrated to be the result of SP-derived TGF β modulating expression of endometrial epithelial cytokine expression [15, 24]. Here, we have yet to define

the active moieties in bovine semen responsible for alterations in endometrial gene expression, or subsequent cellular inflammation in the bovine; however, SP exposure increase expression of *TGFB1* by epithelial and stromal cells which may suggest either amplification of a SP *TGF β* signal or a unique mechanism to induce endometrial inflammation in the cow. More recently, SP-induced IL-17A has been implicated as an intermediary cytokine responsible for endometrial inflammation following insemination [37]. Our data suggest that SP and whole semen increase *IL17A* expression in epithelial and stromal cells, which may aid in cellular inflammation and the influx of $\gamma\delta$ T cells into the endometrium, known to be important in early ruminant pregnancy [38]. However, our data show that no SP-mediated increases of *IL17A* expression were observed in explants or endometrial biopsies following intrauterine infusion; it may be that complex cell–cell interactions regulate SP-mediated *IL17A* expression in tissues where multiple cell types are exposed to SP, or that expression is very acute, as observed by stromal cell increased expression at 30 min post treatment. Expression of IFN ϵ is increased in human cervical cells following exposure to SP [39], while here bovine epithelial or stromal cells only increased *IFNE* expression following exposure to whole semen. It has been hypothesized that elevated IFN ϵ in the reproductive tract confers protection against sexually transmitted pathogens by increasing the number of resident effector immune cells within reproductive tissues of sex workers frequently exposed to semen [40]. Due to the limited exposure of the bovine reproductive tract to semen, it may be that IFN ϵ expression is most related to chronic exposure to semen. Endometrial synthesis of PGF $_{2\alpha}$ and PGE $_2$ are implicated in the modulation of the CL, in addition to their role as classical inflammatory mediators. Here, we demonstrate an increase in both *PTGS2* and *AKR1C4* in epithelial and stromal cells in response to whole semen and SP, which may act as localized inflammatory mediators by increasing prostaglandin synthesis and subsequent vascular dilation and immune cell infiltrate. Indeed, SP itself is rich in prostaglandins (derived from the seminal vesicles) which may be a direct mechanism to increase localized inflammation within female reproductive tissues [41, 42]. In swine, exposure to SP increased expression of prostaglandin synthase enzymes in the endometrium and elevated circulating serum progesterone on day 4 of pregnancy [30, 43]. While it is not clear if SP endometrial prostaglandin modulation is responsible for increased progesterone by altering CL function, we did not find any difference in circulating progesterone following exposure to SP in the cow. While bioactive molecules in SP continue to be described in other species, the method of semen collection used in the current studies should also be considered as it may alter the composition of ejaculated semen. A number of studies in rams, men, and bulls have reported various differences in semen quality, volume, or sperm concentration determined by the method of collection (artificial vagina, electroejaculation, or spontaneous ejaculation), while others report no discernable differences in overall semen quality [44–47]. A proteomic profile of bull SP collected by artificial vagina or electroejaculation reported differential expression of 47 proteins in semen collected by electroejaculation compared to artificial vagina; none of which have been previously identified as molecules involved in the SP induction of endometrial inflammation [48]. Conversely, a similar study in the ram reported no differences in SP proteins [48, 49]. It may be here that the responses observed in our various experimental systems could be altered by using semen collected with an artificial vagina. Similarly, it is not clear if the concentrations of SP used in the current experiments reflect the volume of the ejaculate that would be present in the upper reproductive tract following natural service using bulls. A standard ejaculate

volume may be between 2 and 7 ml in volume, only a fraction of which is likely to arrive cranial of the cervix and be in contact with the endometrium. Similarly, it is not clear if doses of semen utilized for intrauterine infusion are reminiscent of the doses used in *in vitro* experimentation. The distribution of 1 ml of semen throughout the entire uterus may be a significantly lower concentration than those applied to *in vitro* cell cultures. Estimates suggest that the uterine volume of a dairy cow uterus is between 125 and 154 cm 3 [50], resulting in a dose of approximately 0.6 to 0.8% v/v. Future experimentation should also include doses of semen components that are significantly less than those used here.

Seminal plasma is known to increase cellular inflammation in the human cervix, and endometrium of the horse, swine, goats, and rodents (reviewed by Bromfield [51]). While we have not evaluated cellular inflammation in the endometrium of cows exposed to semen or SP, we evaluated the expression of the immune cell marker *PTPRC* (formerly CD45). Interestingly, we did not find an expected increase in the expression of endometrial *PTPRC* following exposure to SP, which would be indicative of increased immune cell infiltrate. Conversely, we found a decrease in *PTPRC* expression when cows were exposed to SP in combination with conventional AI semen, compared to the saline treated controls. While it is unclear whether this downregulation of *PTPRC* is truly representative of decreased cellular inflammation, it is worthy of note that expression in the SP + semen group was not different from treatment with either SP or semen alone. Immunohistochemical evaluation is warranted to confirm an absence of SP induced cellular inflammation in the cow model. The limited SP induction of endometrial inflammation was of surprise in the cow, considering the role of SP in other species and evidence presented here in culture systems. It is possible that the timing of sample collection after treatment here was protracted, or evaluation by other means (such as uterine flushing or cytobrush) is required to capture specific effects of SP-mediated inflammation in the cow. Our *in vitro* data suggest that many observable changes induced by SP exposure occur within 15 min of treatment, and thus SP-induced changes measured may be resolved by 24 h after treatment.

It is unclear whether the developmental environment of the oviduct and uterus of the cow is similar following natural live cover or AI. We propose that SP contributes to alteration in the developmental environment of the bovine endometrium which may subsequently influence early pregnancy. However, there appears to be some discrepancy in our data between *in vitro* culture exposure of endometrial cells to SP and exposure within the cow. Further studies are required to evaluate temporal changes within the cow and to identify the active components of SP responsible for endometrial alterations during early pregnancy. The execution of large field trials is required to ascertain if SP exposure at the time of AI ultimately increases pregnancy success; however, based on the success of AI in the dairy industry it is unlikely to see dramatic increases in conception rates with the addition of SP. However, it may be that SP has a more subtle effect on the phenotype of offspring. Recently, the method of conception was shown to supersede the heritable genetic merit of offspring to produce milk in the first lactation; specifically high genetic merit cows that were derived from IVF had reduced milk production compared to lower genetic merit cows produced by AI [52]. It is likely that these effects are the consequence of having preimplantation embryos develop in a suboptimal *in vitro* environment. It could be hypothesized that SP exposure at conception could further optimize the developmental environment of the preimplantation *in vivo*. Investigations are required to determine the metabolic phenotype

and production value of calves conceived in the presence of SP compared to traditional AI. These studies may extend the economic value of SP supplementation at AI. Ultimately, we hope to supplement AI semen with these SP molecules to develop new AI protocols in which the developmental environment of the preimplantation embryo is optimized for reproductive and phenotypic outcomes in commercial cattle production.

Supplementary data

Supplementary data are available at [BIOLRE](https://doi.org/10.1016/j.biolre.2019.03.010) online.

Supplemental Figure S1. Effect of semen components on endometrial explant gene expression according to stage of estrous cycle. Intact endometrial explant expression of *CSF2*, *IL6*, *CXCL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS2*, *AKR1C4*, and *OXTR* in response to seminal plasma (SP; 2%, 5%, 10%, 20%), 5% whole semen (SM), 5% semen cell pellet (CP), or vehicle medium alone (Veh) after 24 h. Data are presented for individual replicates representing explants isolated from individual cows. Explants derived from cows at stage 1 of the estrous cycle are depicted by open circles, while explants derived from cows at stage 2 of the estrous cycle are depicted as filled circles. Data were log transformed and analyzed using the generalized linear mixed model with pairwise comparisons only made with vehicle treated control. *, *P* value of ≤ 0.05 .

Supplemental Figure S2. Effect of semen components on viability of explants. Intact endometrial explant viability was assessed by measuring LDH activity in supernatants following treatment with seminal plasma (SP; 2%, 5%, 10%, 20%), 5% whole semen (SM), 5% semen cell pellet (CP), or vehicle medium alone (0) for 24 h. Uncultured medium (Veh) was used as a negative control as serum is known to contain elevated LDH. Supernatants collected from explants cultured in the presence of medium containing 1% Triton X-100 were used as a positive control. Data were normalized to the uncultured complete medium (Veh) and are presented as the mean fold change mU/ml LDH activity + S.E.M. from 3–10 independent experiments.

Supplemental Figure S3. Purity of endometrial epithelial and stromal cell cultures. Epithelial cell cultures (A–D) were confirmed as small round cells with epithelial-like morphology, while stromal cell cultures (E–H) had a typical fibroblast-like morphology. To quantify cell purity, flow cytometry using cell specific markers for epithelial (cytokeratin) and stromal (vimentin) cells was performed, in addition to identification of contaminating leukocytes (CD45). Three independent cell isolations were assessed for purity. Endometrial cell cultures contained minimal leukocytes in either epithelial (C; $5 \pm 3\%$ CD45⁺ total cells) or stromal cell cultures (G; $4 \pm 1\%$ CD45⁺ total cells). Stromal cell cultures were $95 \pm 3\%$ vimentin positive (F), while purity for epithelial cell cultures was lower at $56 \pm 16\%$ cytokeratin positive (B). Additionally, viability of epithelial (D) and stromal (H) cells was evaluated using the MTT assay following treatment with seminal plasma (SP), 5% whole semen (SM), 5% semen cell pellet (CP), or vehicle medium alone (Veh). Data from three independent experiments were normalized to vehicle controls and displayed as mean fold change + S.E.M.

Supplemental Figure S4. Effect of intrauterine infusion of semen on day 7 serum progesterone. Serum progesterone was measured in cows 7 days after intrauterine infusion of saline ($n = 11$), conventional semen (12), pooled seminal plasma (SP; $n = 11$), or a combination of conventional semen and SP ($n = 9$). Data are presented as mean ng/ml of progesterone + S.E.M. Data were analyzed using

the generalized linear mixed model with pairwise comparisons. *, *P* value of ≤ 0.05 . T, treatment effect.

Supplemental Table S1. List of qPCR primer sequences for target gene analysis.

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Conflict of Interest: The authors have declared that no conflict of interest exists.

References

- Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ, Robertson SA. Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proc Natl Acad Sci USA* 2014; **111**:2200–2205.
- Norwitz ER, Schust DJ, Fisher SJ. Implantation and the survival of early pregnancy. *N Engl J Med* 2001; **345**:1400–1408.
- Cockburn K, Rossant J. Making the blastocyst: Lessons from the mouse. *J Clin Invest* 2010; **120**:995–1003.
- Kridli RT, Khalaj K, Bidarimath M, Tayade C. Placentation, maternal-fetal interface, and conceptus loss in swine. *Theriogenology* 2016; **85**:135–144.
- Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PL, Melo LF, Ochoa JC, Santos JE, Sartori R. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. *Theriogenology* 2016; **86**:239–253.
- Lima FS, Risco CA, Thatcher MJ, Benzaquen ME, Archbald LF, Santos JE, Thatcher WW. Comparison of reproductive performance in lactating dairy cows bred by natural service or timed artificial insemination. *J Dairy Sci* 2009; **92**:5456–5466.
- Sartori R, Gumen A, Guenther JN, Souza AH, Caraviallo DZ, Wiltbank MC. Comparison of artificial insemination versus embryo transfer in lactating dairy cows. *Theriogenology* 2006; **65**:1311–1321.
- Odhiambo JF, Poole DH, Hughes L, Dejarnette JM, Inskeep EK, Dailey RA. Pregnancy outcome in dairy and beef cattle after artificial insemination and treatment with seminal plasma or transforming growth factor beta-1. *Theriogenology* 2009; **72**:566–571.
- Faulkner LC, Hopwood ML, Wiltbank JN. Seminal vesiculectomy in bulls. II. Seminal characteristics and breeding trials. *Reproduction* 1968; **16**:179–182.
- Lovell JW, Getty R. Fate of semen in the uterus of the sow: Histologic study of endometrium during the 27 hours after natural service. *Am J Vet Res* 1968; **29**:609–625.
- Phillips DM, Mahler S. Leukocyte emigration and migration in the vagina following mating in the rabbit. *Anat Rec* 1977; **189**:45–59.
- Pandya IJ, Cohen J. The leukocytic reaction of the human uterine cervix to spermatozoa. *Fertil Steril* 1985; **43**:417–421.
- De M, Choudhuri R, Wood GW. Determination of the number and distribution of macrophages, lymphocytes and granulocytes in the mouse uterus from mating through implantation. *J Leukoc Biol* 1991; **50**:252–262.
- McMaster MT, Newton RC, Dey SK, Andrews GK. Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. *J Immunol* 1992; **148**:1699–1705.
- Robertson SA, Mau VJ, Tremellen KP, Seamark RF. Role of high molecular weight seminal vesicle proteins in eliciting the uterine inflammatory response to semen in mice. *Reproduction* 1996; **107**:265–277.
- Rozeboom KJ, Troedsson MH, Hodson HH, Shurson GC, Crabo BG. The importance of seminal plasma on the fertility of subsequent artificial inseminations in swine. *J Anim Sci* 2000; **78**:443–448.

17. Alghamdi AS, Foster DN, Troedsson MH. Equine seminal plasma reduces sperm binding to polymorphonuclear neutrophils (PMNs) and improves the fertility of fresh semen inseminated into inflamed uteri. *Reproduction* 2004; 127:593–600.
18. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. *J Immunol* 2012; 188:2445–2454.
19. Yanagimachi R, Chang MC. Infiltration of leucocytes into the uterine lumen of the golden hamster during the oestrous cycle and following mating. *J Reprod Fertil* 1963; 5:389–396.
20. Robertson SA, Mau VJ, Hudson SN, Tremellen KP. Cytokine-leukocyte networks and the establishment of pregnancy. *Am J Reprod Immunol* 1997; 37:438–442.
21. Guerin LR, Moldenhauer LM, Prins JR, Bromfield JJ, Hayball JD, Robertson SA. Seminal fluid regulates accumulation of FOXP3+ regulatory T cells in the preimplantation mouse uterus through expanding the FOXP3+ cell pool and CCL19-mediated recruitment. *Biol Reprod* 2011; 85:397–408.
22. Robertson SA, Guerin LR, Bromfield JJ, Branson KM, Ahlstrom AC, Care AS. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. *Biol Reprod* 2009; 80:1036–1045.
23. Bromfield JJ. A role for seminal plasma in modulating pregnancy outcomes in domestic species. *Reproduction* 2016; 152:R223–R232.
24. Tremellen KP, Seamark RF, Robertson SA. Seminal transforming growth factor beta1 stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus. *Biol Reprod* 1998; 58:1217–1225.
25. Sharkey DJ, Macpherson AM, Tremellen KP, Mottershead DG, Gilchrist RB, Robertson SA. TGF-beta mediates proinflammatory seminal fluid signaling in human cervical epithelial cells. *J Immunol* 2012; 189:1024–1035.
26. Ireland JJ, Murphee RL, Coulson PB. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *J Dairy Sci* 1980; 63:155–160.
27. Borges AM, Healey GD, Sheldon IM. Explants of intact endometrium to model bovine innate immunity and inflammation ex vivo. *Am J Reprod Immunol* 2012; 67:526–539.
28. Turner ML, Cronin JG, Healey GD, Sheldon IM. Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides in vitro via Toll-like receptors TLR2, TLR1, and TLR6. *Endocrinology* 2014; 155:1453–1465.
29. Troedsson MH, Loset K, Alghamdi AM, Dahms B, Crabo BG. Interaction between equine semen and the endometrium: The inflammatory response to semen. *Anim Reprod Sci* 2001; 68:273–278.
30. O'Leary S, Jasper MJ, Warnes GM, Armstrong DT, Robertson SA. Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig. *Reproduction* 2004; 128:237–247.
31. Vishwanath R. Artificial insemination: The state of the art. *Theriogenology* 2003; 59:571–584.
32. Tremellen KP, Valbuena D, Landeras J, Ballesteros A, Martinez J, Mendoza S, Norman RJ, Robertson SA, Simon C. The effect of intercourse on pregnancy rates during assisted human reproduction. *Hum Reprod* 2000; 15:2653–2658.
33. Sjoblom C, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor (GM-CSF) acts independently of the beta common subunit of the GM-CSF receptor to prevent inner cell mass apoptosis in human embryos. *Biol Reprod* 2002; 67:1817–1823.
34. Ziebe S, Loft A, Povlsen BB, Erb K, Agerholm I, Aasted M, Gabrielsen A, Hnida C, Zobel DP, Munding B, Bendz SH, Robertson SA. A randomized clinical trial to evaluate the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) in embryo culture medium for in vitro fertilization. *Fertil Steril* 2013; 99:1600–1609.e2.
35. Block J, Hansen PJ, Loureiro B, Bonilla L. Improving post-transfer survival of bovine embryos produced in vitro: Actions of insulin-like growth factor-1, colony stimulating factor-2 and hyaluronan. *Theriogenology* 2011; 76:1602–1609.
36. Robertson SA, Seamark RF. Granulocyte-macrophage colony stimulating factor (GM-CSF): One of a family of epithelial cell-derived cytokines in the preimplantation uterus. *Reprod Fertil Dev* 1992; 4: 435–448.
37. Song ZH, Li ZY, Li DD, Fang WN, Liu HY, Yang DD, Meng CY, Yang Y, Peng JP. Seminal plasma induces inflammation in the uterus through the gammadelta T/IL-17 pathway. *Sci Rep* 2016; 6:25118.
38. Majewski AC, Tekin S, Hansen PJ. Local versus systemic control of numbers of endometrial T cells during pregnancy in sheep. *Immunology* 2001; 102:317–322.
39. Sharkey DJ, Macpherson AM, Tremellen KP, Robertson SA. Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. *Mol Hum Reprod* 2007; 13:491–501.
40. Abdulhaqq SA, Zorrilla C, Kang G, Yin X, Tamayo V, Seaton KE, Joseph J, Garced S, Tomaras GD, Linn KA, Foulkes AS, Azzoni L et al. HIV-1-negative female sex workers sustain high cervical IFNvarepsilon, low immune activation, and low expression of HIV-1-required host genes. *Mucosal Immunol* 2016; 9:1027–1038.
41. Bielanski W, Rzasa J, Okolski A. Prostaglandins in stallion semen. *Theriogenology* 1982; 17:167–173.
42. Taylor PL, Kelly RW. 19-Hydroxylated E prostaglandins as the major prostaglandins of human semen. *Nature* 1974; 250:665–667.
43. O'Leary S, Jasper MJ, Robertson SA, Armstrong DT. Seminal plasma regulates ovarian progesterone production, leukocyte recruitment and follicular cell responses in the pig. *Reproduction* 2006; 132:147–158.
44. Jimenez-Rabada P, Ramon M, Garcia-Alvarez O, Maroto-Morales A, del Olmo E, Perez-Guzman MD, Bisbal A, Fernandez-Santos MR, Garde JJ, Soler AJ. Effect of semen collection method (artificial vagina vs. electroejaculation), extender and centrifugation on post-thaw sperm quality of Blanca-Celtibérica buck ejaculates. *Anim Reprod Sci* 2012; 132: 88–95.
45. Moore RW. A comparison of electro-ejaculation with the artificial vagina for ram semen collection. *N Z Vet J* 1985; 33:22–23.
46. Hovav Y, Almagor M, Yaffe H. Comparison of semen quality obtained by electroejaculation and spontaneous ejaculation in men suffering from ejaculation disorder. *Hum Reprod* 2002; 17:3170–3172.
47. Austin JW, Hupp EW, Murphee RL. Comparison of quality of bull semen collected in the artificial vagina and by electroejaculation. *J Dairy Sci* 1961; 44:2292–2297.
48. Rego JP, Moura AA, Nouwens AS, McGowan MR, Boe-Hansen GB. Seminal plasma protein profiles of ejaculates obtained by internal artificial vagina and electroejaculation in Brahman bulls. *Anim Reprod Sci* 2015; 160:126–137.
49. Marco-Jimenez F, Vicente JS, Viudes-de-Castro MP. Seminal plasma composition from ejaculates collected by artificial vagina and electroejaculation in Guirra ram. *Reprod Domest Anim* 2008; 43:403–408.
50. Baez GM, Barletta RV, Guenther JN, Gaska JM, Wiltbank MC. Effect of uterine size on fertility of lactating dairy cows. *Theriogenology* 2016; 85:1357–1366.
51. Bromfield JJ. Review: The potential of seminal fluid mediated paternal-maternal communication to optimise pregnancy success. *Animal* 2018; 12:s104–s109.
52. Siqueira LGB, Dikmen S, Ortega MS, Hansen PJ. Postnatal phenotype of dairy cows is altered by in vitro embryo production using reverse X-sorted semen. *J Dairy Sci* 2017; 100:5899–5908.