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Bull seminal plasma stimulates in vitro production of TGF-β, IL-6 and IL-8 from bovine endometrial epithelial cells, depending on dose and bull fertility

Thanapol Nongbua[a,b,1], Yongzhi Guo[a,1], Theodoros Ntallaris[a], Marie Rubér[c], Heriberto Rodriguez-Martinez[c], Patrice Humblot[a], Jane M. Morrell[a,*]

[a] Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden
[b] Faculty of Veterinary Sciences, Mahasarakham University, Maha Sarakham, Thailand
[c] Department of Biomedical and Clinical Sciences (BKV)/BKH-Obstetrics&Gynaecology, Linköping University, Linköping, Sweden

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ABSTRACT

Seminal plasma (SP) regulates immune responses in the female reproductive tract through specific cytokines. It is not known whether SP from high fertility bulls (H) differs from SP from low fertility bulls (L). In this study, the cytokine response of bovine endometrial epithelial cells (bEEC) in culture was investigated after challenge with SP from two bulls of below average (L) or three bulls of above average fertility (H). The bEECs were challenged with 1% or 4% SP from L- or H-fertility bulls (L1, L4, H1, H4, respectively) or 1% or 4% PBS as control (C1, C4) for 72 h. The culture media were analysed for concentrations (pg/million cells) of transforming growth factor beta (TGF-β1, TGF-β2 and TGF-β3) by Luminex, and Interleukin 6 and 8 (IL-6, IL-8) by ELISA. Challenge significantly affected production of TGF-ß1, TGF-ß2 and IL-8 compared to controls and was affected by bull fertility ($p < 0.0001$), SP concentration ($p < 0.0001$) and their interaction ($p < 0.0001$). A higher production of TGF-β1, TGF-β2 and IL-8 ($p < 0.0001$), and also IL-6 ($p < 0.01$), resulted from challenge with high doses of SP, being higher for L than H ($p < 0.05$). For TGF-β3, fertility of bull ($p < 0.05$). For TGF-B3, fertility of bull ($p < 0.05$) and the interaction between fertility and concentration of SP were significant ($p < 0.01$). In conclusion, 4% SP from L bulls stimulated more TGF-β1, TGF-β2, TGF-β3, IL-6 and IL-8 production than SP from H bulls, indicating that stimulation of the endometrium is relevant for fertility. Seminal plasma from high fertility bulls seems to affect cytokine production *in utero* positively in inseminated cows.

1. Introduction

The liquid portion of semen, seminal plasma (SP), promotes sperm motility and serves as a medium for transporting the spermatozoa into the female genital tract in some species (Bromfield, 2016; Maxwell et al., 2007; Poiani, 2006). It is predominantly produced by the accessory sex glands and also contains small amounts of testicular and epididymal fluid (Bromfield, 2016). The major components of bovine SP include peptidase proteins, cytokines, enzymes, antioxidants, hormones, ions, sugar and lipid (Juyena and Stelletta, 2012).

Bovine SP has a major function in stimulating and supporting spermatozoa through the provision of nutrients, providing a protective environment and enhancing sperm motility in the female (Poiani, 2006; Juyena and Stelletta, 2012). In addition, SP protects spermatozoa against immune attack in the female reproductive tract (Poiani, 2006; Robertson, 2005). It contributes to sperm capacitation and also to fertilization by promoting sperm movement, regulating pH, supporting nutrition, and preventing acrosome exocytosis (Rodriguez-Martinez et al., 2011; Maxwell et al., 2007; Poiani, 2006). Thus, bovine SP can both inhibit and stimulate sperm function and fertility (Maxwell et al., 2007b). Active moieties in the seminal fluid cooperate with endometrial epithelial cells to promote synthesis of cytokines that modulate the inflammatory response, and facilitate embryo tolerance, expansion and implantation in mammals (Robertson, 2005; Robertson and Sharkey, 2001). However, the mechanisms by which these processes occur are unclear. The specific effects of bovine SP on bovine endometrial cells are not known although SP from low fertility bulls had an adverse effect on cell viability in culture in a dose dependent

* Corresponding author.
E-mail address: jane.morrell@slu.se (J.M. Morrell).
[1] Joint first authors.

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fashion (Nongbua et al., 2018).

Within the bovine artificial insemination (AI) industry, differences in fertility between bulls have traditionally been attributed to sperm quality while the contribution of bovine SP to fertility has been largely ignored. The assumption is that SP in the original ejaculate is diluted considerably by the addition of extender when preparing insemination doses. However, SP is not physically removed from the semen doses and small quantities (0.8–12 % of the original volume) are introduced into the uterus during AI (Nongbua et al., 2019). It would not normally be present in the uterus of cattle after natural mating (Alghamdi et al., 2009) since semen is deposited in the vagina of bovids. However, seminal plasma biomolecules are adsorbed on the spermatozoa and accompany them all the way to the site of fertilization, prompting interaction with the epithelium lining the reproductive tract (Rodríguez -Martinez et al., 1998). Thus, the differences in fertility among individuals with apparently similar sperm quality could be due to variations in bovine SP composition and its effect on both spermatozoa and the female genital tract. Differences in specific proteins between bulls with low or high fertility based on bovine SP composition (Morrell et al., 2015; Killian et al., 1993) and protein expression (D'Amour et al., 2010; Peddinti et al., 2008; Moura et al., 2006) have been reported. However, there are differing opinions on whether or not SP has an effect in inseminated cows. Odhiambo et al. (2009) were not able to find a difference in fertility after administering SP to cows at insemination (unless fertility was less than 50 %), whereas the administration of TGF-ß1 tended to increase pregnancy rates. Similarly, Pfeiffer et al. (2012) did not observe an effect on pregnancy rates when inseminated cows were exposed to a vasectomized bull although, somewhat surprisingly, exposure to a bull with a surgically diverted penis improved pregnancy rates. In another study, SP administered at the time of AI with conventional semen reduced pregnancy rate at day 32 and tended to reduce calving rate, although no effect was observed if sexed semen was used (Ortiz et al., 2019). Therefore, in the context of AI, observations on a possible effect of SP vary considerably.

Since the endometrial epithelial cells are exposed to, and interact with, SP following AI (Bromfield, 2016; Robertson, 2005), the effect of the interaction may differ between bulls of different fertility. Therefore, the objective of the present study was to investigate specific cytokine production by bovine endometrial epithelial cells (bEEC) in culture in response to challenge with SP from bulls of high or low fertility. The cytokines chosen were members of the transforming growth factor-β (TGF-β) family, TGF-β1, TGF-β2 and TGF-β3, which are thought to be some of the key signaling moieties in SP (Robertson, 2007), and also IL-6 and IL-8, the latter being considered to be one of the key elements in the immune response to pathogens (Donofrio et al., 2010).

2. Materials and methods

2.1. Preparation of bovine SP

Ejaculates collected by artificial vagina were available from bulls of known fertility according to a fertility index used by the breeding company (Viking Genetics, Skara, Sweden). The fertility index is based on the 56-day non-return rate, i.e. cows not returning to oestrus at 56 days after AI are assumed to be pregnant. This index was adjusted to take into account factors such as time of year, farm, age and parity of cow, and inseminator (Rodriguez-Martinez, 2003), based on a cohort study. The scores are then normalized with bulls of average fertility in the cohort scoring 100. The other bulls in the cohort are given a score that is either higher than average or lower than average. Samples were available from three above average fertility bulls (fertility index > 103; H) and two below average fertility bulls (< 93; L). Aliquots were centrifuged at 1800 x g for 10 min to pellet the spermatozoa. The supernatant was removed and checked microscopically for the presence of spermatozoa. If no spermatozoa were observed, the seminal plasma was frozen in 1 mL aliquots and stored at −20 °C. Centrifugation was repeated as necessary until the SP was free of spermatozoa. Since there were several bulls of known fertility, SP from individual bulls was used separately to determine if any effect seen was induced by all samples; there was no pooling of ejaculates i.e. the seminal plasma was from individual bulls.

2.2. Preparation, isolation and culture of bEEC

Bovine uteri were obtained from a local abattoir. The ovaries and their structures were examined carefully to determine the stage of the oestrous cycle (pro-oestrus, oestrus, metoestrus or dioestrus) from the size, color and hemorrhagic appearance of corpora lutea (Ireland et al., 1980). Only uteri from dioestrus cows without any visible signs of pathology, such as inflammation, were used. Cell preparation was performed within 1 h of receiving the uterus at the slaughterhouse, after transport to the laboratory. Preparation, isolation and culture of bEEC were performed using a method adapted from Charpigny et al. (1999) as previously described (Chanrot et al., 2017a,b; Nongbua et al., 2018). The endometrial tissue was cut into 2 − 3 mm pieces and transferred to enzymatic dissociation solution consisting of Dulbecco's phosphate buffer saline 250 mL (PBS; Gibco, Invitrogen, CA), Collagenase IV 250 U/mL (Sigma, St. Louis, MO), hyaluronidase 250 U/mL (Sigma, St. Louis, MO), 2% bovine serum albumin (Sigma, St. Louis, MO). After incubation at 39 °C for 2.5 h in a water bath, the suspension was filtered through sterile gauze to remove the debris and undigested tissue. The filtrate was re-filtered using a specific 40 µm cell strainer to separate fibroblasts and epithelial cells. The epithelial cells on the top of the filter were washed with 10 mL sterile PBS and were centrifuged at 120 x g for 5 min. The cells were resuspended in complete medium (Dulbecco's Modified Eagle's Medium F-12 [DMEM]; Sigma, St. Louis, MO) with 10 % fetal bovine serum (FBS), 0.5 % of Liquid Media Supplement (I3146, Sigma, St. Louis, MO), 2.5 mM of L-glutamine (Gibco, Invitrogen, CA), 50 U/mL penicillin and streptomycin (Gibco, Invitrogen, CA), 0.01 mg/mL gentamicin (Sigma, St. Louis, MO) and 100 U/mL nystatin (Sigma, St. Louis, MO). The DMEM did not contain phenol red. The epithelial cells were incubated at 39 °C in 5% CO_2 for 5 days until cell confluence was reached (approximately 90 % of flask). The purity of epithelial cells in culture from passage 3 (> 98 %) were confirmed by flow cytometry (Becton Dickinson, San Jose, CA) after addition of anti-Cytokeratin 18 antibody (cat ab 668; Abcam, UK) and anti-Vimentin V9 antibody (cat ab175473; Abcam, UK) immunofluorescence staining (Guo et al., 2019).

2.3. Challenge with bovine SP

The bEEC from passage 3 were cultured in flasks with 25 cm^2 surface area (BD Falcon) and incubated at 39 °C in 5% CO_2. Cells were subcultured to produce sufficient numbers for the experiment. Cell numbers per batch varied from 3.0–5.5 × 10^5. In each batch, the cells were split equally among 7 flasks for subculture. At 72 h, the attached cells in one flask were collected and counted to give the total cells before challenge (TBC). The cells were counted as follows: bEEC were detached using trypsin express (Gibco). Aliquots of the cell suspension were stained with trypan blue (Sigma) and counted in a Burker hemocytometer (Nongbua et al., 2018). Cell viability was classified according to staining: unstained (live) or stained (dead cells, both floating and detached cells) (Louis & Siegel, 2011). The evaluator was unaware of the treatment groups during the evaluation.

The cells in the remaining 6 flasks were subsequently challenged with either 75 µL or 300 µL SP from low (L) or high (H) fertility bulls, in a final volume of 7 mL (representing 1% or 4% of total volume medium in flask), or with the corresponding volumes of PBS (Gibco, Invitrogen, CA) as a control (C). Thus, the treatments L1, L4, H1, H4, C1, C4 were applied to each batch of cells (biological replicates). Cells were available from eight uteri; cells from 5 uteri were used for two batches each. These doses of SP were chosen to represent the amounts of SP that

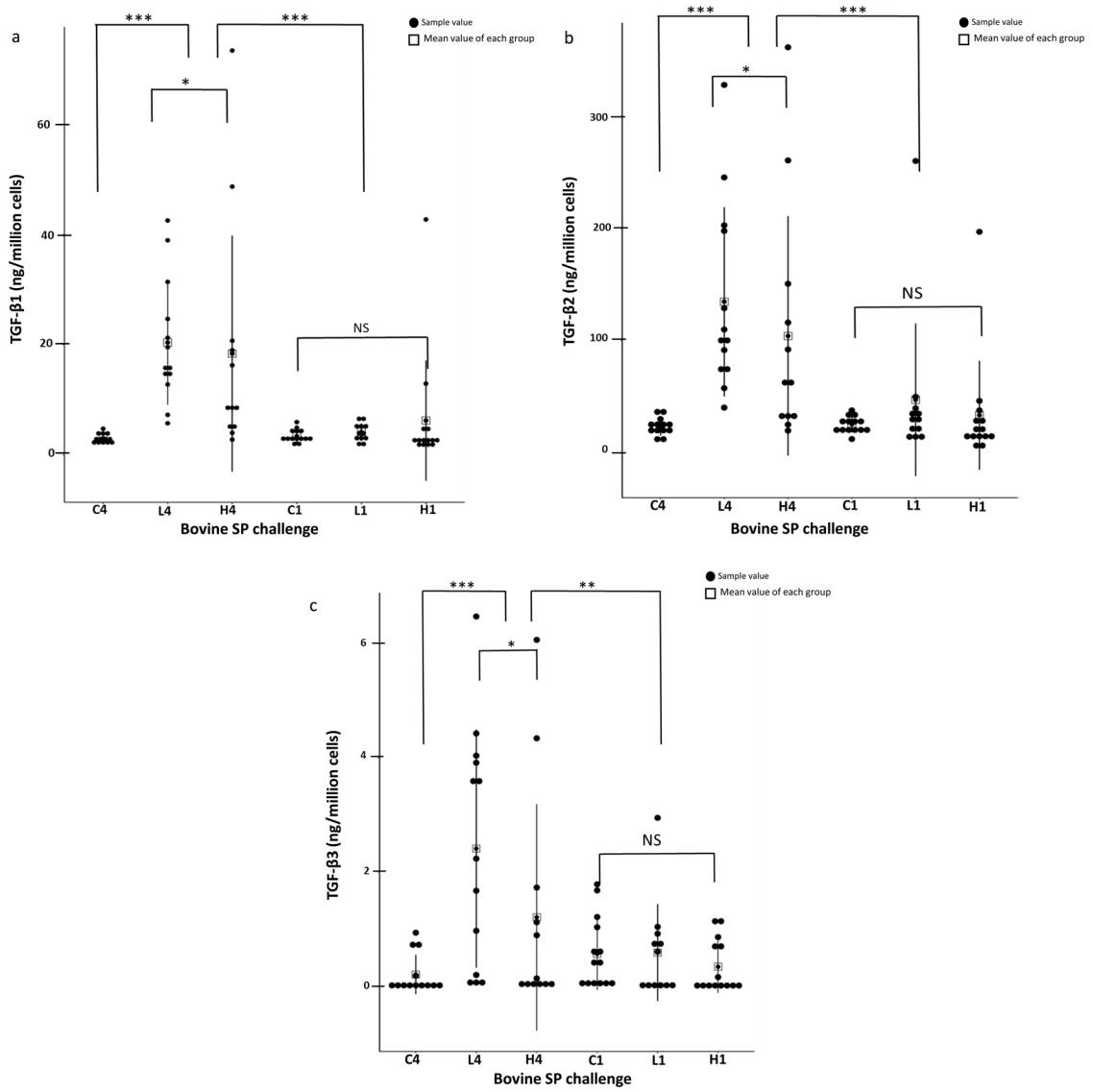

Fig. 1. Effect of challenging bovine endothelial epithelial cells in vitro with two concentrations of bovine seminal plasma from bulls of low and high fertility on production of cytokines TGF-β1 (a), TGF-β2 (b) and TGF-β3 (c).
Note: C1, C4 = controls [phosphate buffered saline, 1% (75 µL) and 4% (300 µL), respectively]; L1, L4 = seminal plasma from low fertility bulls at 1%, 4% respectively; H1, H4 = seminal plasma from high fertility bulls, at 1% and 4% respectively.

commonly occur in insemination doses, calculated from actual ejaculates, with the proviso that not more than 4% of the culture medium could be replaced by SP without depriving the cells of essential nutrients (Nongbua et al., 2018). It was not possible to include a positive control in this study since several dosages would have been required, with and without SP, making the experiment too cumbersome to run. Many different types of positive controls could have been used, each of them potentially inducing a specific/different response, which would have made it difficult to choose a relevant control before doing the experiment. Previous experiments from our laboratory show that LPS and SP induce a different type of inflammatory response (Chanrot et al., 2017a,b, Guo et al., 2019).

2.4. Cytokine analysis

The bEEC were incubated for a further 72 h before collecting the cells by centrifugation (215 x *g* for 5 min.) to remove debris and dead cells. The attached bEECs were detached by adding 5 mL trypsin (Tryple express; Gibco), and counted to give the total cells after challenge (TAC), as reported previously (Nongbua et al., 2018). The culture medium was cleaned by centrifuging the collected medium at 8000 rpm for 5 min at room temperature before transferring the supernatant to a new tube for analysis of the following cytokines: Transforming growth factor beta (TGF-β1, TGF-β2 and TGF-β3) by Luminex (MILLIPLEX™ TGFß Magnetic Bead 3 Plex Kit - Immunology Multiplex Assay SDS, Cat#TGFB-64K-03, for wide animal reactivity, Merck Millipore, Sweden), Interleukin 6 (IL-6) (Thermo Fisher Scientific Catalog # ESS0029) and Interleukin 8 (IL-8) by ELISA (Bovine IL-8 (CXCL8) ELISA development kit, MABTECH AB, Sweden). All assays were performed in 96-well plates following the respective manufacturer's guidelines. The concentration of each cytokine was calculated as ng/million cells.

For TGF-β1, TGF-β2 and TGF-β3, the samples were acidified (pH < 3) with 8 µL of HCl and diluted 1:30 v/v with assay buffer and sample diluent before analysis. A cytokine standard curve, comprising six standard points, was built for each cytokine with the highest standard point at 10,000 pg/mL and the lowest standard point at 9.8 pg/mL. Serum matrix (SM), provided in the kits, was used to mimic the composition of the seminal environment in the standard, control and blank measurements. Two controls provided in the kits were added in singlets. Following sonication, bead solution was added to each well for

incubation at 4 °C in the dark, for 18 h. After incubation, the plate was emptied using a multiscreen vacuum manifold (Merck Millipore), washed twice, detection antibody added, incubated at RT in darkness for 60 min, before streptavidin–phycoerythrin addition and further incubated for 30 min. After washing, the plates were run on a Luminex 200 T M (Luminexcorp, Austin, TX, USA) with xPONENT software version 3.1.7 (Luminex corp) for acquisition and Masterplex 2010 version 2.0.0.68 (Mirai Bio Group, San Francisco, CA, USA) for data analysis. The median fluorescent intensity was analysed using a 5-parameter logistic curve-fitting to calculate the concentrations of the cytokines in the samples.

The limit of detection for the IL-8 kit is 8–800 pg/mL; the analytical sensitivity of IL-6 kit is < 78.1 pg/mL, and the assay range was 78.1 − 5000 pg/mL. Before unit conversion, the Elisa data from this study were all within the detection range.

2.5. Statistics

Data were analysed using the mixed model in SAS® (Proc Mixed, SAS® 9.3, USA). Fertility of bull, concentration of SP, and their interaction were fixed parts of the model, with cytokine response as variable parameter. Cow and cow interaction with replication were used as random factors. Post-hoc comparisons were adjusted for multiplicity using Tukey´s correction test, and the SAS Contrast and estimate options were used to analyze individual differences. A $p < 0.05$ was considered statistically significant.

3. Results

Note: all values are presented as Least square means ± group SEM.

3.1. TGF-ß1, TGF-ß2 and TGF- ß3 production

The effect of challenge with bovine SP on production of the cytokines TGF-ß1, TGF-ß2 and TGF- ß3 is shown in Fig. 1. Challenge had a significant effect on TGF-ß1 (Fig. 1a) and TGF-ß2 production (Fig. 1b), depending on bull fertility ($p < 0.0001$) and SP concentration ($p < 0.0001$). The interaction between these factors was also significant ($p < 0.0001$). However, there were no differences in TGF-ß1 or TGF-ß2 between L1, H1, C1 and C4. There was a significantly higher ($p < 0.001$) production of both TGF-ß1 and TGF-ß2 from L4 and H4 than from C4. A higher production of TGF-ß1 resulted from challenge with L4 than H4. Similarly, a higher production of TGF-β2 resulted from challenge with L4 than H4.

For TGF-β3 production (Fig. 1c), fertility of bull ($p < 0.05$) and the interaction between fertrility and concentration of SP were significant (< 0.01). The production of TGF-β3 was higher in L4 than H4 ($p < 0.05$), higher in L1 than H1 ($p < 0.01$), higher in L1 than in C1 and higher in L4 than in C4 ($p < 0.01$).

3.2. IL-6 production

Challenge with SP had a significant effect on IL-6 production (Fig. 2a) that was influenced by concentration of SP ($p < 0.001$) and fertility of bull ($p < 0.001$). The production of IL-6 was lower after challenge with L1 SP compared to H1 SP. Challenge with SP from low fertility bulls or high fertility bulls resulted in a higher production of IL-6 ($p < 0.001$) than controls. There were no differences in IL-6 production after challenge with L1 and H1 compared to C1 and C4. A higher production of IL-6 ($p < 0.05$) resulted from challenge with L4 and H4; challenge with L4 SP differed from C1, C4 and H1 ($p < 0.05$).

3.3. IL-8 production

There were no differences in IL-8 production (Fig. 2b) after challenge with L1 and H1 (18.0 ± 5.1 and 18.4 ± 4.7 ng/million cells, respectively) compared to C1 and C4 (17.5 ± 4.7 and 17.1 ± 4.9 ng/million cells, respectively). A higher production of IL-8 ($p < 0.0001$) resulted from challenge with L4 and H4 (54.8 ± 4.9 and 44.6 ± 5.1 ng/million cells, respectively). Challenge with L4 SP differed from H4 ($p < 0.05$).

4. Discussion

In this experiment, bovine SP was shown to affect production of the five cytokines TGF-β1, TGF-β2, TGF-β3, IL-6 and IL-8 by bEEC, depending on the level of challenge and the fertility of the donor bulls. Moreover, production of these cytokines was greater after challenge with a high dose of SP from a low fertility bull than from a high fertility bull. It has been shown previously that fertility is associated with the proportions of SP proteins, for example, the concentration of heparin-binding proteins in beef bull semen (Bellin et al., 1994). These heparin-binding proteins, in addition to being involved in sperm capacitation and the acrosome reaction (Chandonnet et al., 1990), are implicated in immunoregulation in the female reproductive tract (Juyena and Stelletta, 2012; Moura et al., 2007).

These results are in general agreement with the findings of Rizo et al. (2019) who observed that bovine semen was able to modulate endometrial gene expression *in vitro,* partly through the action of TGF-ß. However, they considered that other bioactive elements in semen might alter the endometrial environment. Other studies by the same group reported that SP affected expression of a variety of cytokines (including IL-6 and TGF-ß) by endometrial cells either *in vitro* or *in vivo* (Ibrahim et al., 2019). The difference between our results and these *in vitro* observations, as well as the AI studies reported previously, is that SP from bulls of known high or low fertility was used in our experiment. Different effects were observed depending on whether the SP came from high or low fertility bulls. This observation may help to explain the different effects reported in the literature.

A study on the proteins present in SP from high and low fertility bulls identified 1159 proteins in total (Viana et al., 2018). There were differences between the two classes of bull, with 50 proteins being more abundant in high fertility bulls than low fertility bulls, and 29 being more abundant in low fertility bulls than high fertility bulls (Viana et al., 2018). Similarly, a study on the metabolome of bull semen identified differences in metabolites such as fructose and 2-oxo-glutaric acid between bulls of high and low fertility (Velho et al., 2018). Therefore, several components of SP appear to be differentially present in SP from bulls of high or low fertility and could explain the differential effect on bEEC cells.

Cytokines are believed to have a signaling function and, also, to act as adhesion mediators, facilitating binding to receptors (Koh et al., 2018). Cytokine binding to receptors on target cells in the cervix and uterus activates changes in gene expression, leading to modifications in tissue structure and function preparatory to placentation (Robertson, 2005). Placentogenesis in cattle requires the presence of a competent elongated blastocyst and an endometrium that is primed to receive it, which has been achieved through effective intracellular communication mediated by cytokines. The activity of TGF beta super-family members is believed to play a pivotal role in such endometrial remodelling (Sugawara et al., 2010), which has been extensively studied in humans and mice. These proteins play diverse and fundamental roles in female reproduction include follicular development, ovulation, oocyte competence, decidualization, in particular during implantation, pregnancy, embryonic development and uterine development (Qinglei, 2014). Development of mouse embryos with *tgfb1*gene mutant are arrested at the morula stage, but can be rescued by maternal TGFβ-1, leading to perinatal survival of these mice (Letterio et al., 1994). In vitro treatment of preimplantation stage embryos with TGFB-1 increases total numbers of cells in expanded and hatching blastocysts (Lim et al., 1993). The *Tgfb2* transcript is expressed in luminal and glandular epithelium during the mouse peri-implantation period (Das et al., 1992),

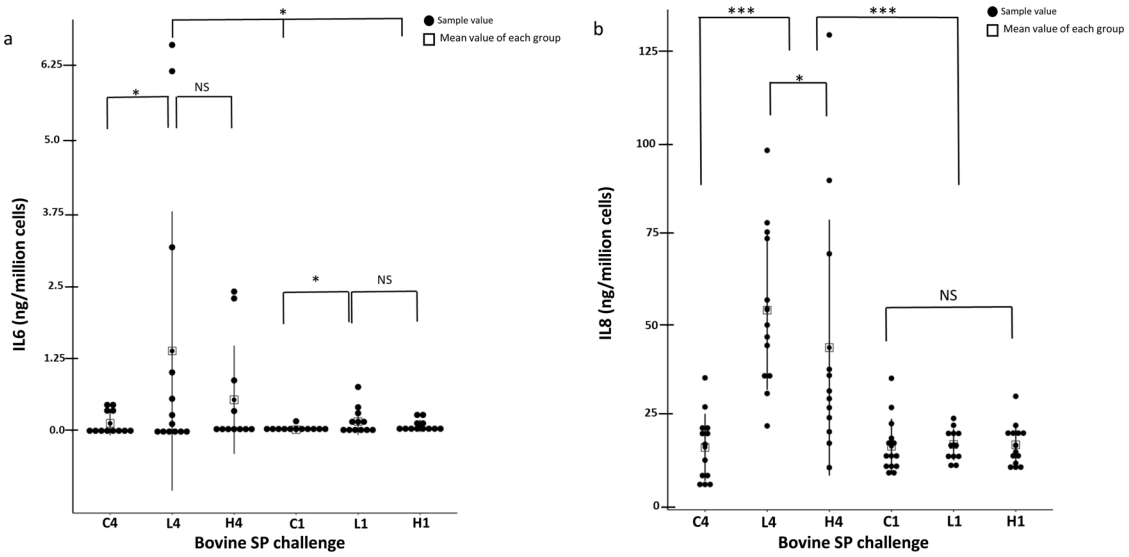

Fig. 2. Effect of challenging bovine endothelial epithelial cells in vitro with two concentrations of bovine seminal plasma from low and high fertility bulls on production of cytokines IL-6 (a) and IL-8 (b).
Note: C1, C4 = controls [phosphate buffered saline, 1% (75 μL) and 4% (300 μL), respectively]; L1, L4 = seminal plasma from low fertility bulls at 1%, 4% respectively; H1, H4 = seminal plasma from high fertility bulls, at 1% and 4% respectively.

and uterine Tgfb2 or *Tgfb3* mRNA persisted in ovariectomized mice. *Tgfb3* transcripts are detected in myometrial cells and in the vascular smooth muscle cells during the peri- and post implantation periods. Although mRNA for both TGFβ-1 and II receptors was detected throughout preimplantation development in bovine embryos (Roelen et al., 1998), the role of TGFβ super-family members during implantation is still unclear in ruminants. In our study, high doses of SP stimulated TGFB-1 and 2 expression from bEECs in culture, suggesting that the stimulation of bEECs by SP *in vivo* could be conducive to embryo implantation.

Apart from their importance as an early signal for placentogenesis, these cytokines may also play a part in triggering the immune response to rid the uterus of microorganisms potentially introduced during mating/insemination. Other cytokines, in particular IL-β and TNFα, participate in complement activation and the acute phase response to pathogens (Harju et al., 2005; Healy et al., 2014; Splichal and Trebichavsky, 2001), whereas secretion of IL8 during uterine infection or inflammation attracts granulocytes to the endometrium (Donofrio et al., 2010). In a study comparing serum and uterine washings from healthy cows and those with subclinical endometritis, uterine washings contained significantly higher levels of IL-6, together with IL-10 and Hp in the group with subclinical endometritis compared to controls (Brodzki et al., 2015). Certainly, inadequate pathogen eradication from the uterus resulting in prolonged inflammatory signaling is considered to be detrimental to fertilization and conception (LeBlanc, 2014; Sheldon et al., 2017).

Endometrial cells in culture, such as in the present study, can be used to model the processes occurring in response to artificial insemination as well as in response to inflammatory stimuli. Endometrial cell cultures were shown to secrete IL-6 and IL-8 when challenged with IL-1α, suggesting that the latter accentuates the cells´ response to damage (Healey et al., 2014). When uterine epithelium and stromal cells in culture were challenged with lipopolysaccharide and IL-1b, expression of the IL-6 cytokine gene was significantly upregulated, along with CXCL8/IL-8, IL-1A and IL-1B (Koh et al., 2018; Guo et al., 2019). In the present model of bovine endometrial epithelial cells, LPS increased IL-6 and Il-8, and production of IL-8 occurred after challenge of bEEC with bovine Herpes virus type 4 (Chanrot et al., 2017a,b). Exposure of bEECs to LPS was also associated with the under-expression of many genes coding for immuno-modulatory molecules, including TGFβ (Guo et al., 2019). In contrast to the results presented here, the expression of all TGFβ mRNAs was increased by SP, consistent with its priming role in activating immuno-modulatory mechanisms (Guzeloglu-Kayisili et al., 2009). A strong rise in TGFβ production and mRNA expression was observed following exposure of cells to all SP samples, but the increase was more pronounced for low than for high fertility bulls. The physiological significance of this difference deserves further investigation. The present model was sufficiently sensitive to determine the effects associated with the deposition of small quantities of SP in the uterus, as would occur during AI in the cow.

In a previous study from our group, challenge with 4% bovine SP was shown to have a detrimental effect on bEEC viability, particularly from low fertility bulls (Nongbua et al., 2018), whereas challenge with 1% SP did not have a detrimental effect. The results of the present study were in agreement with this previous report, where there appeared to be a dose-dependent effect apart from the bull fertility-dependent effect of bovine SP on bEEC in culture. Some cytokines are known to have a negative effect on cell growth (Guzeloglu-Kayisili et al., 2009) but it is not known whether the cytokines studied here are likely to have contributed to the loss of viability of bEEC observed in our previous study.

These results lend support to the speculation that the fertility of a bull is the result of the interaction between SP, spermatozoa and the uterus of the recipient cow. The SP is not removed when preparing conventional bovine semen doses but is diluted by the addition of the semen extender. The proportion of bovine SP present in the insemination dose varies depending on the sperm concentration of the original ejaculate and the number of spermatozoa required. In the present study, the bEEC were challenged with 1 or 4% SP, well within the range of 0.8%–12% of the AI dose estimated in other studies (Hering et al., 2014; Bromfield, 2016; Nongbua et al., 2018). Thus, the challenge in this study is equivalent to the challenge experienced locally by bEEC after deposition of a single semen dose into the uterus during AI. Therefore, it seems likely that bovine SP exerts a differential effect on fertility via cytokine production from the uterine epithelium. In the future it may be possible to modulate the effect of SP from low fertility bulls by removing the SP prior to insemination, for example using colloid centrifugation (Nongbua et al., 2017).

In conclusion, challenging bEEC in culture with high concentrations of SP stimulated more cytokine production than low concentrations or PBS; 4% SP from low fertility bulls stimulated more TGF-β1, TGF-β2,

TGF-β3, IL-6 and IL-8 production than SP from high fertility bulls. Unfavorable effects may be mediated specifically through the increase of the pro-inflammatory IL-6 and IL-8, which might be associated with a variety of cell damage. These results may help to explain potential uterine responses in inseminated cows.

Declaration of Competing Interest

The authors have no conflicting interests to declare.

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