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Seminal Plasma Induces Prostaglandin-Endoperoxide Synthase (PTGS) 2 Expression in Immortalized Human Vaginal Cells: Involvement of Semen Prostaglandin E2 in PTGS2 Upregulation[1]

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ABSTRACT

Inflammation of the cervicovaginal mucosa is considered a risk factor for HIV infection in heterosexual transmission. In this context, seminal plasma (SP) may play an important role that is not limited to being the main carrier for the virions. It is known that SP induces an inflammatory reaction in the cervix called postcoital leukocytic reaction, which has been associated with promotion of fertility. The mechanisms by which SP triggers this reaction, however, have not been clearly established. Previously we reported the expression of prostaglandin-endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase 2 (COX-2), in human vaginal cells in response to toll-like receptor (TLR) ligands and other proinflammatory stimuli. In this study, we demonstrate that SP induces transcriptional and translational increase of COX-2 expression in human vaginal cells and cervicovaginal tissue explants. Furthermore, SP potentiates vaginal PTGS2 expression induced by other proinflammatory stimulants, such as TLR ligands and a vaginal mucosal irritant (nonoxynol-9) in a synergistic manner. SP-induced PTGS2 expression is mediated by intracellular signaling pathways involving MAPKs and NF-κB. Using fractionation and functional analysis, seminal prostaglandin (PG)-E_2 was identified as a one of the major factors in PTGS2 induction. Given the critical role of this PG-producing enzyme in mucosal inflammatory processes, the finding that SP induces and potentiates the expression of PTGS2 in cervicovaginal cells and tissues has mechanistic implications for the role of SP in fertility-associated mucosal leukocytic reaction and its potential HIV infection-enhancing effect.

NF-kB, prostaglandin E_2, PTGS2/COX-2, seminal plasma, sexually transmitted infections, toll-like receptors (TLRs), vaginal inflammation

INTRODUCTION

Mucosal inflammation of the female lower genital tract is regarded as an important factor favoring acquisition of HIV-1 infection via vaginal intercourse [1–3]. The sites of inflammation are enriched with HIV target immune cells and may have breaches in the mucosal epithelial barrier, both of which facilitate HIV acquisition [4]. Seminal plasma (SP) is the main vector for HIV in sexual transmission, but SP is not only a carrier for HIV [5, 6]. In vitro studies have shown that SP may both inhibit and facilitate HIV infection. Inhibitory effects of SP include antiviral activity of the semen cationic polypeptides, suppression of HIV binding to a subset of dendritic cells, protection of HIV-target cells by semenogelin, inactivation of HIV by SP-reactive oxygen species, and SP-induced increase in transepithelial resistance [7–12]. In contrast, SP may facilitate HIV infection in several ways. Semen-derived enhancer of viral infection (SEVI) amyloid fibrils have been shown to promote virions' attachment to target cells [13]. In cervicovaginal mucosa, SP induces upregulation of CCL20, a chemokine involved in attracting Langerhans cells to the epithelium, a phenomenon associated with HIV transmission [14]. Neutralization of the vaginal acidic pH, deleterious to virion survival, is an additional factor that increases the chances of HIV infection [15].

From in vivo studies, it is also known that semen causes the so-called postcoital inflammatory response, or leukocytic reaction [16, 17]. Presence of seminal plasma in the mammalian reproductive tract results in an immediate and dramatic influx of immune cells to the cervix and an increase in the level of proinflammatory cytokines and chemokines [18]. These changes are believed to play a role in facilitating conception [18, 19]. In immortalized cervical cells, SP has been reported to stimulate proinflammatory cytokines such as IL-8, IL-6, CSF2, CCL2, GM-CSF, and CCL20 [14, 20, 21].

SP has also been shown to induce prostaglandin-endoperoxide synthase 2 (*PTGS2*), also known as cyclooxygenase 2 (*COX-2*), in cervical adenocarcinoma cells in vitro and ectocervical cells in vitro and in vivo [16, 20, 21]. *PTGS2* codes for an enzyme that is ubiquitously expressed in inflammatory settings [22]. It catalyzes the rate-limiting step in the synthesis of prostaglandins. A major PTGS2 product, prostaglandin (PG) E_2, is essential in inflammation-related tissue changes [23]. It induces vasodilation and increases vascular permeability, resulting in massive influx of immune cells, including HIV target cells, to the sites of inflammation. Elevated levels of PGE_2 result in chemoattraction and activation of immune cells and are associated with visible signs of acute inflammation [24]. Previously, we identified PTGS2 as a biomarker for vaginal inflammation. We have reported the induction of vaginal PTGS2 in response to diverse proinflammatory stimulants, such as TNF-α, microbial ligands, and cell membrane-damaging surfactants [25]. In this study, we demonstrate that SP causes PTGS2 upregulation in human vaginal cells, confirming the inflammatory potential of SP and suggesting a possible role in HIV-1 transmission. We also identify PGE_2, abundantly present in SP, as one of the major factors responsible for PTGS2 induction in vaginal cells.

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MATERIALS AND METHODS

Materials

Microbial ligands, Pam_3CSK_4 (Pam) and Lipoteichoic acid (LTA), and the immunomodulatory compound imiquimod (IMQ) were purchased from Invivogen (San Diego, CA). Nonoxynol-9 (N-9) was a kind gift from OrthoMcNeil Corporation (Raritan, NJ). 16,16-dimethyl (dm) PGE_2 and PGE_2 receptor antagonist AH-6809 were purchased from Cayman chemical (Ann Arbor, MI). Signal transduction inhibitors Bay11-7082, SB 202190, and U0126 were purchased from Calbiochem (Billerica, MA). Amicon Centrifugal Filter Devices with cutoffs of 100, 50, and 30 kD were purchased from Millipore Corporation (Billerica, MA). The primary antibodies used in Western blots were anti-COX-2 (PTGS2; Abcam Inc., Cambridge, MA), anti-beta actin (ACTB; BD Transduction Labs, Chicago, IL), anti-phos p38MAPK (Biosource, Grand Island, NY), anti-phos ERK1/2, anti-p38MAPK and anti-ERK 1/2 (Cell Signaling Technology, Boston, MA), anti-IkB-α and anti-NF-kB/p65 (RE-LA; Santa Cruz Biotechnology, Santa Cruz, CA).

Semen Collection and Preparation

Human semen was obtained from healthy, normozoospermic men enrolled in an Eastern Virginia Medical School Institutional Review Board (IRB)-approved semen donation program. Semen was left at room temperature for about 1 h to allow liquefaction to occur. The samples were then centrifuged at 4000 rpm for 20 min at room temperature. Samples were either used the same day or aliquoted and stored at −80°C. In some experiments, several samples were pooled. Some samples were purchased from Lee Biosolutions (St. Louis, MO).

Seminal Plasma Fractionation and Heat Treatment

To test for the heat lability of PTGS2-stimulating factor(s), SP was incubated at 95°C for 10 min. Molecular-weight fractionation of SP was done by sequential filtration of SP diluted in growth medium with Amicon Centrifugal Filter Devices with cutoffs of 100, 50, and 30 kD. All the retained and flow-through fractions were stored at −80°C until used.

Cell Culture

The vaginal epithelial cell line VK-2/E6E7 was a gift from Dr. Raina Fichorova (Brigham and Women's Hospital, MA). This cell line was derived from epithelial cells of vaginal mucosal tissue from a 32-yr-old premenopausal woman undergoing anterior-posterior repair and was demonstrated to have structural and functional properties similar to those of their parental primary cells [26]. VK-2 cells were cultured to ~80% confluency in 100- or 35-mm plates using keratinocyte serum-free medium (Gibco, Invitrogen, Grand Island, NY) supplemented with bovine pituitary extract (50 μg/ml) and epidermal growth factor at 0.1 ng/ml, penicillin-streptomycin (1%) and $CaCl_2$ (0.4 mM). Medium was replenished every other day.

Cytotoxicity

Cells were grown in 96-well plates and incubated for 24 h with all the test compounds. Viability was estimated using CellTiter 96 AQ_{ueous} One solution Cell Proliferation assay (Promega, Madison, WI).

Electrophoresis and Immunoblotting

Cells were washed three times with cold PBS and lysed with Laemmli loading buffer followed by boiling for 10 min to obtain total cellular protein. Nuclear and cytoplasmic fractions were isolated as described elsewhere [27] with minor modifications. Briefly, the cells were rinsed with ice-cold PBS and scraped with buffer containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 3 mM $CaCl_2$, and 0.5% Triton X-100. The samples were centrifuged at $1000 \times g$ for 5 min. The supernatant and the pellet were the cytoplasmic and nuclear fraction respectively.

These fractions were lysed using Laemmli loading buffer as mentioned above. Nuclear and cytoplasmic fractions were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membrane (Immobilon-P; Millipore). Nonspecific binding was blocked for 1 h in PBS containing 0.1% Tween-20 and 5% nonfat dry milk for 1 h. The membrane was incubated overnight at 4°C with specific primary antibodies. After incubation, the membrane was washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (Invitrogen). Immunodetection was performed using enhanced chemiluminiscent Western blotting detection reagents (Amersham, GE Healthcare, Piscataway, NJ). To monitor protein loading for immunoblotting, the membrane was stripped, and ACTB, or total p38MAPK or ERK 1/2, was detected. Immunoblots were visualized using FluorChem Q Imaging System (Alpha Innotech, San Leandro, CA). Quantification of the blots was done using Image J (National Institutes of Health, Bethesda, MD). Intensity of PTGS2 bands was quantified in arbitrary units by normalizing to ACTB.

RNA Extraction and Quantitative RT-PCR

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) followed by purification using RNeasy columns (Qiagen, Valencia, CA) according to the manufacturer's recommendations.

For quantitative PCR analysis, RNA (1 μg) was converted to cDNA using a combination of random primers (reverse transcription system; Promega). PCR amplification was performed using the LightCycler FastStart DNA Master SYBR Green I (Roche, Indianapolis, IN) kit, according to the manufacturer's recommendations. *GAPDH* was used as the internal standard for PCR. The specific primers used were as follows:

PTGS2: Forward 5′-TGAGCATCTACGGTTTGCTG-3′ and
Reverse 5′-TGCTTGTCTGGAACAACTGC-3′;
GAPDH: Forward 5′-GAGTCAACGGATTTGGTCGT-3′ and
Reverse 5′-GATCTCGCTCCTGGAAGATG-3′.

The thermocycler parameters were 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec and 55°C for 5 sec and 72°C for 15 sec. Expression of *PTGS2* mRNA was normalized using *GAPDH*. Relative gene expression was calculated by dividing the normalized expression in SP-treated cells by that of cells with growth medium only.

Quantitation of PGE_2

SP PGE_2 quantitation was done by multiple reaction monitoring (MRM) and liquid chromatography method using an ACQUITY UPLC system (Waters Corp., Milford, MA) at the Proteomics and Metabolomics Shared Resource, Lombardi Comprehensive Cancer Center of Georgetown University. (See description of procedure in Supplemental *Materials and Methods*, available online at www.biolreprod.org.)

Each SP sample was prepared in triplicates, and three injections of each preparation were performed to assess reproducibility. The mass spectrometry data from the UPLC-TOFMS were processed using the TargetLynx (Waters Corp.).

Ectocervical and Vaginal Tissue Culture and Sample Processing

Ectocervical and vaginal tissues were obtained from premenopausal women (36 and 44 yr old, respectively) undergoing surgery because of benign gynecological conditions (fibroids and rectocele/cystocele). Proper consent was obtained according to an Eastern Virginia Medical School IRB-approved protocol. The mucosal and submucosal layers were dissected from the musculature. The tissues were cut into pieces 5 mm in diameter with 2–4 mm in thickness. They were acclimated at 37°C with 5% CO_2 on cell culture inserts in RPMI 1640 (Invitrogen) supplemented with 10% FBS and penicillin-streptomycin (1%). Subsequently, the tissues were subjected to different treatments. For immunoblotting, proteins were solubilized by homogenization of tissues in Laemmli loading buffer.

Immunohistochemistry (IHC) staining was performed with paraffin-embedded tissues fixed in 4% buffered formalin (Fisher Scientific, Hanover Park, IL). Briefly, the slides were deparaffinized, dehydrated, and rehydrated as per standard procedures. This was followed by antigen retrieval in citrate buffer (pH 6.2) at high temperature. Thereafter, the slides were cooled and washed with PBS. This was followed by incubation with anti-COX-2 (PTGS2) primary antibody (Abcam) at 1:500 dilution. Nonspecific binding was blocked by 1.5% goat serum. The slides were washed and incubated with goat anti-rabbit biotinylated secondary antibody followed by ABC reagent (Vectastain Labs, Burlingame, CA). The antigen was localized by incubation with AEC chromogen (ScyTek Labs, Logan, UT).

Data Analysis and Statistics

Statistical analysis and graphic presentation (Student *t*-test, Pearson correlation) were done using GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA). *P*-values less than 0.05 were regarded statistically significant. Statistical significance is indicated in the legends to figures. Quantification of immunoblots was done using Image J software (National

Institutes of Health). Densitometric readings of PTGS2 signals on immunoblots were normalized to ACTB used as loading control and expressed in arbitrary units.

RESULTS

Effects of SP on Viability of Vaginal Cells

Human vaginal (VK-2/E6E7) cells were incubated with SP at different concentrations ranging from 0.1% to 30%. Viability was evaluated 24 h postincubation. A noticeable decrease in cell viability (down to 83% of medium control) was observed at a SP concentration of 20%, followed by a further drop in viability (to 65% of control) at a SP concentration of 30% (Fig. 1). Therefore, only SP concentrations $\leq$10% were used in the following experiments.

SP Induces a Dose-Dependent Increase in PTGS2 Expression in Human Vaginal Cells

Changes in *PTGS2* mRNA and protein levels in vaginal cells in response to SP were assayed by quantitative RT-PCR and immunoblotting. Expression of *PTGS2* followed a dose-dependent relationship with SP. The quantitative RT-PCR analysis revealed that *PTGS2* mRNA expression increased 9-fold after treating the cells with 5% SP and 16-fold after treatment with 10% SP, compared to control (Fig. 2A). *PTGS2* mRNA induction was corroborated by a similar dose-dependent increase in PTGS2 protein expression, as observed by immunoblotting (Fig. 2B).

SP Activates NF-kB and MAPK Pathways That Participate in PTGS2 Expression in Vaginal Cells

Depending on cell type and stimulus, different intracellular signaling pathways are shown to be involved in inflammation and PTGS2 expression. Nuclear factor kB (NF-kB) pathway is considered of central importance in inflammation and PTGS2 induction [28]. Activation of NF-kB in VK-2 cells stimulated with SP was demonstrated by rapid (within 30 min) degradation of NF-kB inhibitor IkB-α in cytoplasm accompanied by release and nuclear translocation of REL A (also known as NF-kB/p65; Fig. 3A). In addition, activation of mitogen-activated kinases (MAPK) p38 and ERK 1/2 was demonstrated by their phosphorylation, which occurred in VK-2 cells within 15 min of being incubated with SP (Fig. 3A). To confirm the involvement of these pathways in PTGS2 expression, VK-2 cells were treated with the pathway-specific

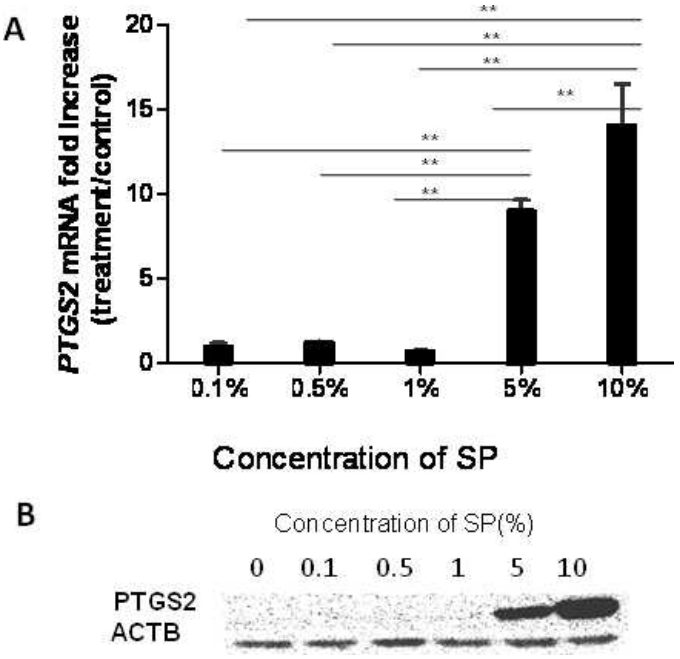

FIG. 2. SP stimulates transcriptional and translational PTGS2 expression in VK-2 cells in a dose-dependent manner. VK-2 cells grown in 35-mm plates were treated with 0.1–10% SP for 24 h and assayed for PTGS2 expression. **A**) *PTGS2* mRNA upregulation was assayed by quantitative RT-PCR. *GAPDH* was used as a normalizing gene. Results are expressed as the mean ± SD of multiple experiments. $^{**}P < 0.01$. **B**) PTGS2 protein expression was assayed by immunoblotting. ACTB was used as a loading control

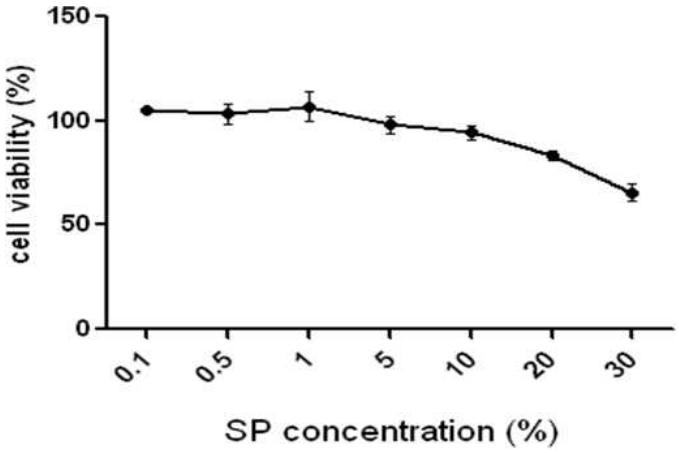

FIG. 1. Dose-dependent cytotoxicity of SP. VK-2 cells were treated for 24 h with SP at concentrations ranging from 0.1% to 30% v/v in culture medium. Viability was measured by CellTiter 96 AQ_{ueous} One solution Cell Proliferation assay (Promega). Viability of SP-treated cells was estimated in relation to the viability of the cells treated with culture medium alone taken as 100%. Results are expressed as mean ± SD of three independent experiments.

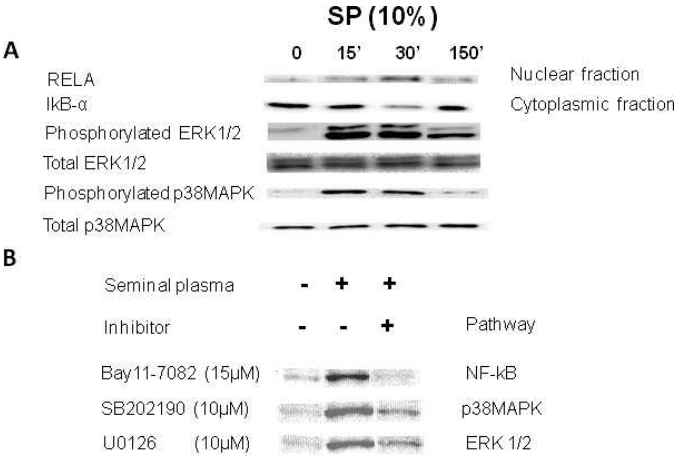

FIG. 3. MAPK and NF-kB pathways are involved in SP-induced PTGS2 expression. VK-2 cells grown in 100-mm plates were treated with 10% SP for indicated time. **A**) Nuclear fractions were assayed for REL A translocation; cytoplasmic fractions were assayed for IkB-α degradation; whole cell lysates were assayed for phosphorylation of ERK1/2 and p38MAPK by immunoblotting. **B**) Immunoblots of PTGS2 expression in VK-2 cells treated with SP in the presence or absence of the indicated pathway-specific inhibitors for 6 h; ACTB was used as a loading control.

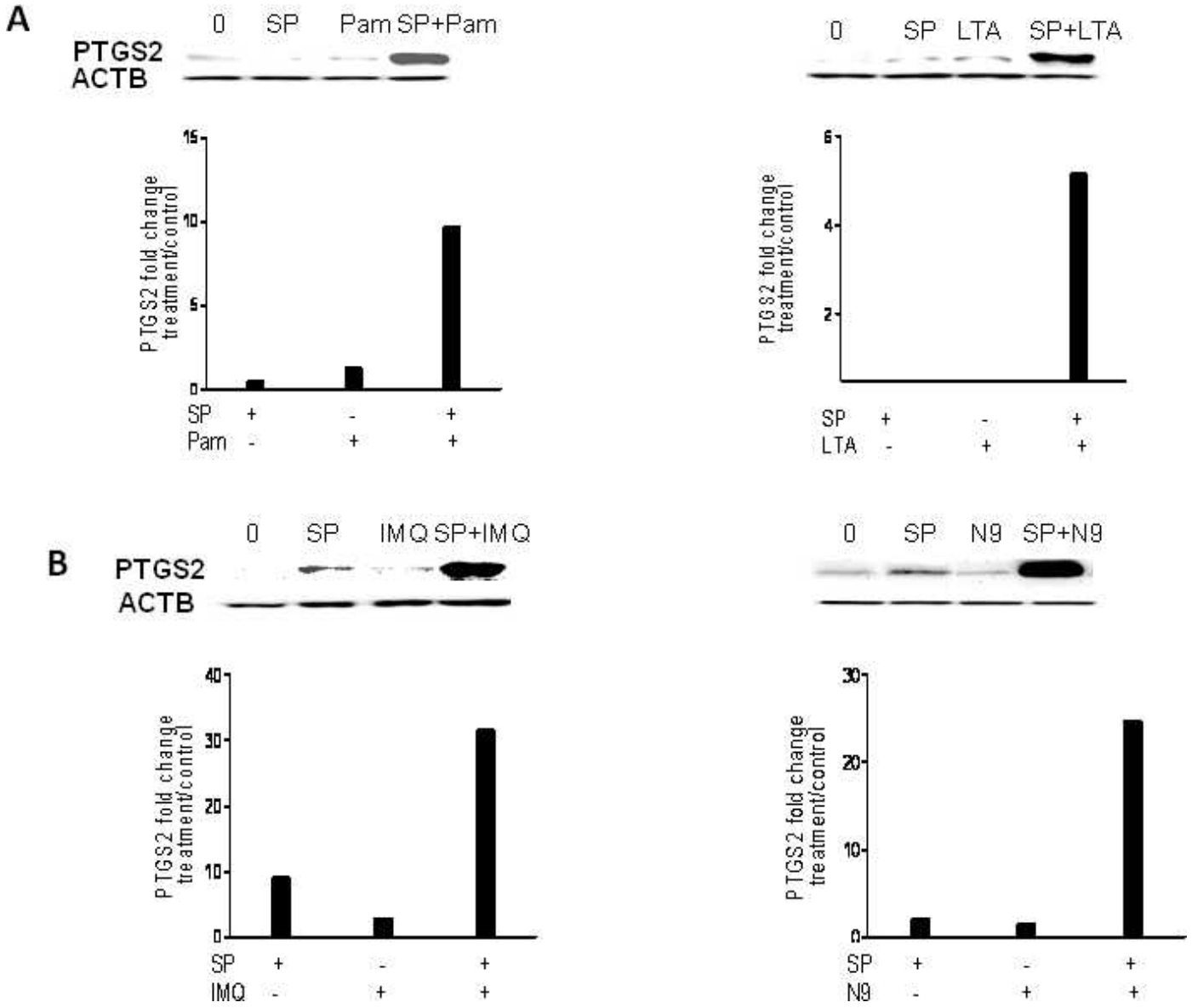

FIG. 4. SP induces expression of PTGS2 in synergy with other pro-inflammatory stimuli. VK-2 cells grown in 35-mm plates were treated with SP and proinflammatory stimuli for 24 h. Expression of PTGS2 was assayed by immunoblotting, ACTB was used as a loading control (**A**). Bacterial lipopeptides Pam and LTA were used at concentrations of 5 and 10 μg/ml, respectively, in the presence of 1% SP. **B**) Inflammatory compounds N-9 and IMQ were used at concentrations of 6 and 10 μg/ml in the presence of 3% SP. The graph below each blot shows the band intensity of PTGS2 expressed in arbitrary units, normalized to ACTB. Values were obtained from densitometric readings using Image J.

inhibitors Bay11-7082 (NF-kB pathway), SB202190 (p38MAPK), and U0126 (ERK 1/2). Presence of each of the inhibitors abolished expression of PTGS2 (Fig. 3B), confirming the pathway involvement in SP induced PTGS2 expression by vaginal cells.

SP Potentiates PTGS2 Expression Caused by TLR Ligands and N-9

We previously demonstrated that TLR ligands Pam, LTA, and IMQ and the proinflammatory surfactant N-9 induce PTGS2 expression in VK-2 cells [25, 29]. In this study, we evaluated the effect of SP on PTGS2 expression in the presence of these compounds. Suboptimal concentrations of the compounds and SP dilutions were selected so that individual treatments of cells would result in low to negligible expression of PTGS2. However, when the compounds and SP were combined at these concentrations, PTGS2 protein expression was much higher than a mere sum of the PTGS2 induced by either of the treatments (Fig. 4). The observed synergistic effect of SP and TLR ligands implies that exposure to SP could potentiate inflammatory responses of the female vaginal epithelium to microbial antigens.

SP from Different Individuals Shows Variability in Its Capacity to Induce PTGS2 Expression

To evaluate interindividual variability in SP capacity to induce PTGS2, VK-2 cells were treated with SP from 12 different donors. Figure 5 shows a considerable interindividual variation in *PTGS2* mRNA induction as demonstrated by quantitative RT-PCR. A mean of 24-fold increase in *PTGS2*

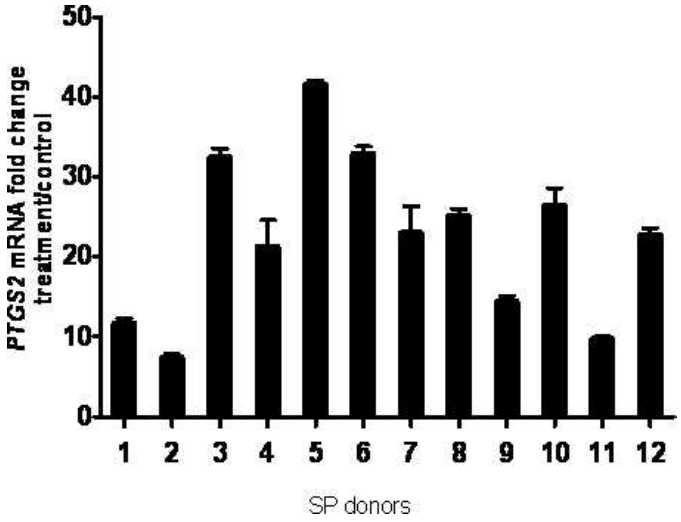

FIG. 5. PTGS2-inducing capacity of SP varies among individuals. VK-2 cells grown in 35-mm plates were treated with 10% SP from 12 donors for 24 h. *PTGS2* mRNA was assayed by quantitative RT-PCR. Data represent the extent of *PTGS2* mRNA upregulation by SP from individual donors. Treatments were performed in triplicates. *GAPDH* was used as a normalizing gene.

expression was observed across the group with donor-to-donor variation ranging from 7.4- to 41.6-fold compared to control (Fig. 5). Variations (although not as high in magnitude) in the *PTGS2* stimulatory effect were also observed between semen samples from the same donor obtained on different days (data not shown).

PGE_2 Is a Major Factor in SP-Mediated Stimulation of PTGS2

In an initial step to characterize the factor(s) responsible for PTGS2 induction, SP was heated for 10 min at 95°C. Figure 6A demonstrates that heat treatment drastically decreased the capacity of SP to induce PTGS2 in VK-2 cells, suggesting that the SP factor(s) responsible for increasing PTGS2 production was heat labile.

Next, SP was fractionated on the basis of the the molecular weights of its constituents using Amicon Centrifugal Filters with cutoffs of 100, 50, and 30. VK-2 cells were then treated with filter-retained and flow-through fractions. Fractions that passed through the filters with a cutoff of 30 kDa remained active in PTGS2 induction. In contrast, the retained fractions did not cause PTGS2 expression. Notably, although flow-through lower-molecular-weight fractions were free from higher-molecular-weight proteins, the separation was not complete for the retained fractions. Small proteins, similar to those contained in the flow-through fractions, were still present in the retained fractions (observed by gel electrophoresis), while PTGS2-stimulating activity was lost in them, suggesting that PTGS2 was induced by small nonprotein molecules present in the flow-through fraction. PGE2 is a biologically active, heat-labile, small molecule (molecular weight = 352.5 Da) that is present in high quantity in SP [30]. Importantly, PGE_2 is known to stimulate PTGS2 expression in various cells, including cervical adenocarcinoma cells [21]. We hypothesized that PGE_2 could be the factor responsible for PTGS2 induction. To test this hypothesis, VK-2 cells were treated with PGE_2 receptor antagonist AH 6809, which has equal affinity to multiple PGE_2 receptors [31]. SP induction of PTGS2 was completely inhibited in the presence of the PGE_2 receptor antagonist. Furthermore, we confirmed the stimulatory activity

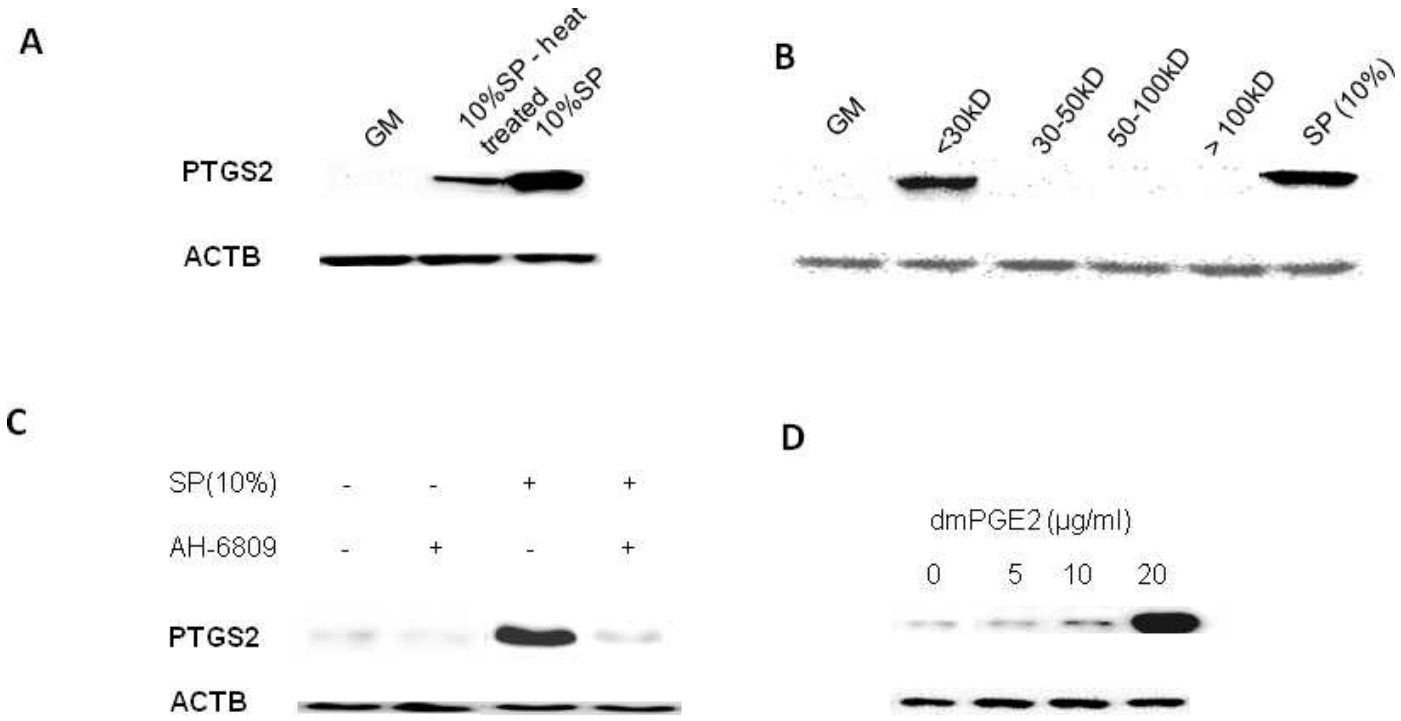

FIG. 6. PGE_2 is an important factor responsible for PTGS2 induction by SP. VK-2 cells grown in 35-mm plates were treated for 24 h with (**A**) heated 10% SP, (**B**) SP fractions corresponding to a varying range of molecular weights, (**C**) 10% SP in the presence of PGE_2 receptor antagonist AH-6809 (60 µM), and (**D**) dm PGE_2 at concentrations of 5, 10, and 20 µg/ml. PTGS2 expression was assayed by immunoblotting; ACTB was used as a loading control. GM, growth medium.

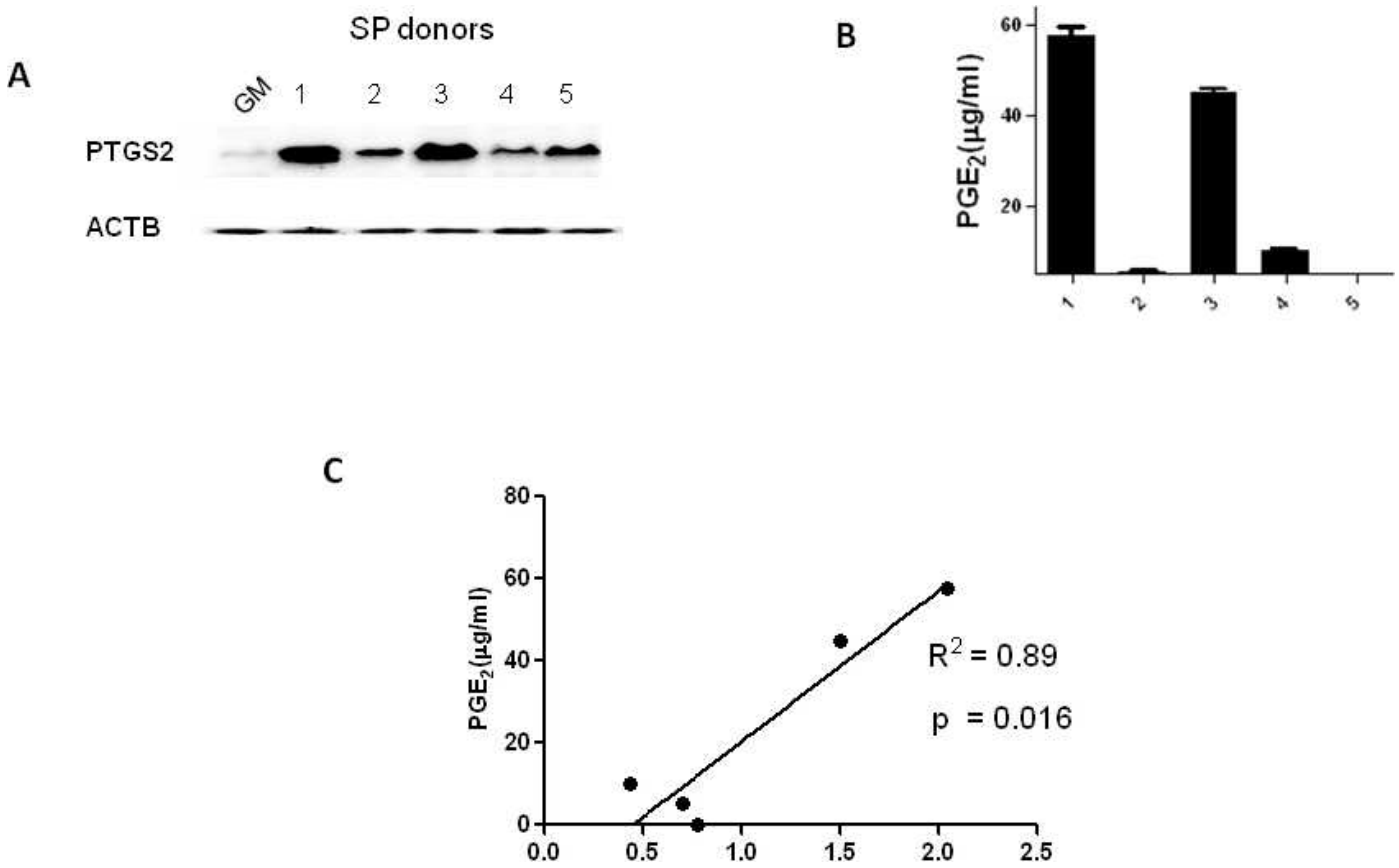

FIG. 7. PTGS2 induction correlates with the levels of PGE_2 in SP. SP samples with highest and lowest PTGS2-inducing activity were selected for PGE_2 quantitation. **A**) PTGS2 protein expression in VK-2 cells grown in 35-mm plates was assayed by immunoblotting; ACTB was used as a loading control. GM, growth medium. **B**) PGE_2 concentration was quantified by liquid chromatography/mass spectrometry. **C**) Correlation between PTGS2 expression was quantified from the immunoblot (using Image J) and SP PGE_2 level.

of PGE_2 by testing $dmPGE_2$ (a stable form of PGE_2), which caused a dose-dependent increase in PTGS2 expression in VK-2 cells (Fig. 6D).

PGE_2 Levels in Seminal Plasma Correlate with Seminal Plasma Ability to Stimulate PTGS2 Expression in VK-2 Cells

PGE_2 was quantified in several individual SP samples showing distinctly high or low PTGS2 induction capacity using liquid chromatography (Fig. 7, A and B). PTGS2 protein levels in VK-2 cells were evaluated by PTGS2 immunoblot band intensities normalized to ACTB. A positive correlation (Pearson correlation coefficient $R^2 = 0.89$, $P = 0.016$) between the concentration of PGE_2 and PTGS2 levels was observed (Fig. 7C), supporting the hypothesis that PGE_2 in SP is implicated in PTGS2 induction in vaginal epithelial cells.

SP Induces PTGS2 Expression in Ectocervical and Vaginal Tissue Explants

Next, we verified the expression of PTGS2 in response to SP in ectocervical tissue explants. Cultured cervical tissues were treated with pooled SP at 75% in medium for 24 h. A clear increase in PTGS2 protein expression was observed by IHC staining (Fig. 8A) and was further corroborated by immunoblotting using anti-PTGS2 antibodies (Fig. 8B). Similar results indicating increase in PTGS2 expression were obtained for vaginal explants treated with 75% SP for 24 h (Fig. 9).

DISCUSSION

Earlier, we reported that diverse proinflammatory stimuli cause expression of PTGS2 in human vaginal epithelial cells [25, 29]. PTGS2 (or COX-2) is an inducible enzyme that is essential in promoting inflammation [32–34]. It catalyzes the rate-limiting step in the synthesis of prostaglandins. A major PTGS2 product, PGE_2, is considered to be the primary culprit of inflammation-related changes in tissues [23]. Here, we report that SP causes transcriptional and translational dose-dependent induction of PTGS2 in human vaginal epithelial cells.

SP induction of PTGS2 in the female genital tract has been demonstrated in endometrial tissues of horses and pigs and in human cervical adenocarcinoma cells [21, 35, 36]. More recently, SP-induced PTGS2 expression was also observed in normal cervical biopsies as part of an inflammatory-like response postulated to be necessary for promotion of fertility [16, 20].

One of the central pathways involved in cellular inflammatory responses and PTGS2 upregulation is the NF-kB signaling pathway. In addition, different MAPKs may also be activated in response to proinflammatory stimuli and engaged in PTGS2 expression [37]. Previously, we have reported that NF-kB and MAPK pathways are involved in PTGS2 expression in vaginal cells in response to the proinflammatory spermicide N-9 [25]. We also found that these pathways are implicated in PTGS2 upregulation via TLRs stimulated by microbial pathogens (unpublished results). Here, we demonstrate that the same

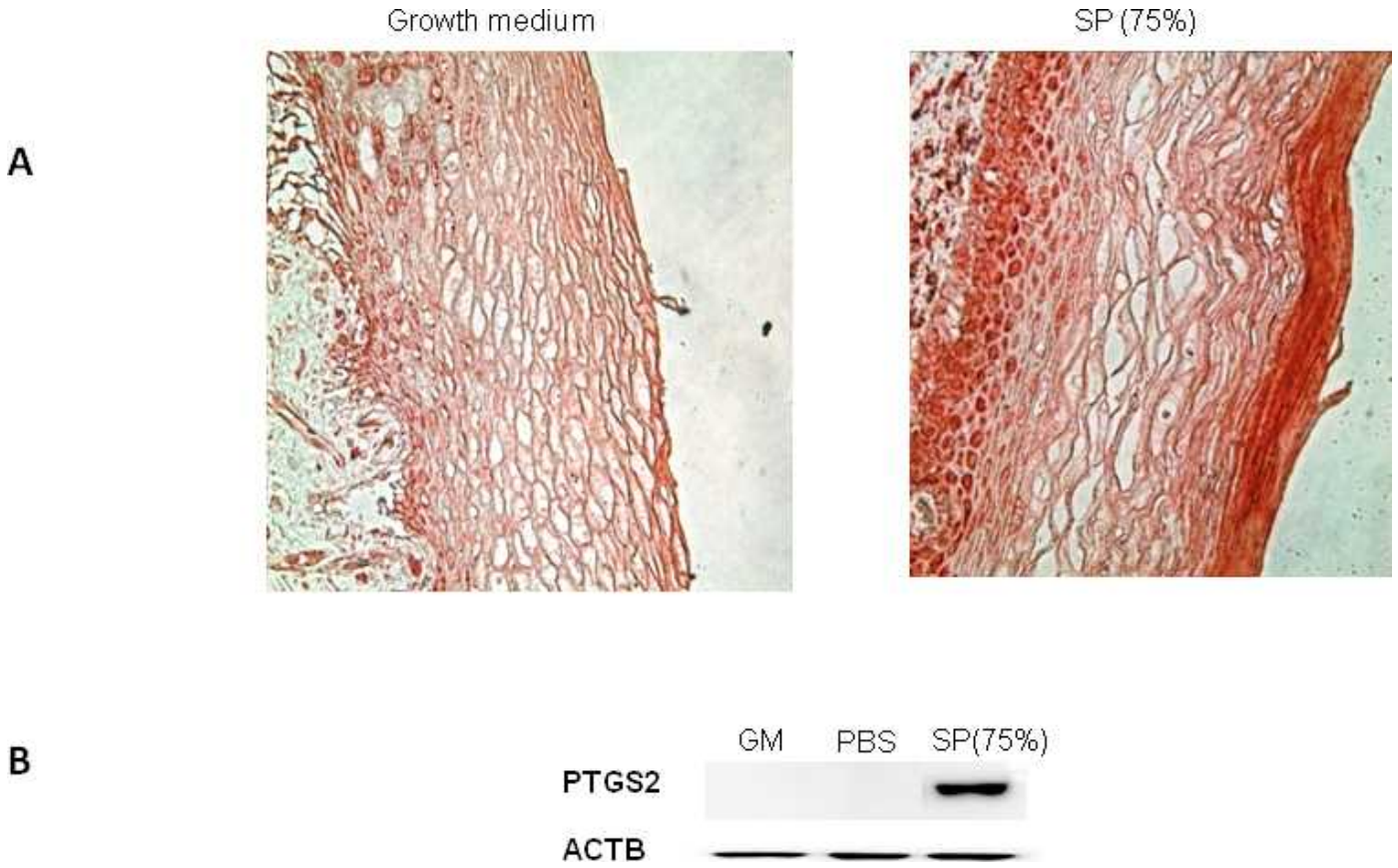

FIG. 8. SP induces PTGS2 in ectocervical tissue explants in culture. Ectocervical tissues were treated with SP (75%) for 24 h. PTGS2 expression was assayed by (**A**) IHC and (**B**) immunoblotting; ACTB was used as a loading control. GM, growth medium. Original magnification ×400.

signaling pathways, NF-kB, p38MAPK, and ERK1/2, are also activated by SP and that their activation is responsible for PTGS2 induction (Fig. 3).

Given the commonality in induction pathways, we wanted to evaluate the impact of SP on PTGS2 expression in the presence of other proinflammatory stimuli commonly present in the vaginal environment. We found that SP acts in synergy with TLR ligands, such as Pam, LTA, and IMQ and the vaginal spermicide N-9. We demonstrated that PTGS2 expression induced by these compounds is strongly potentiated by SP

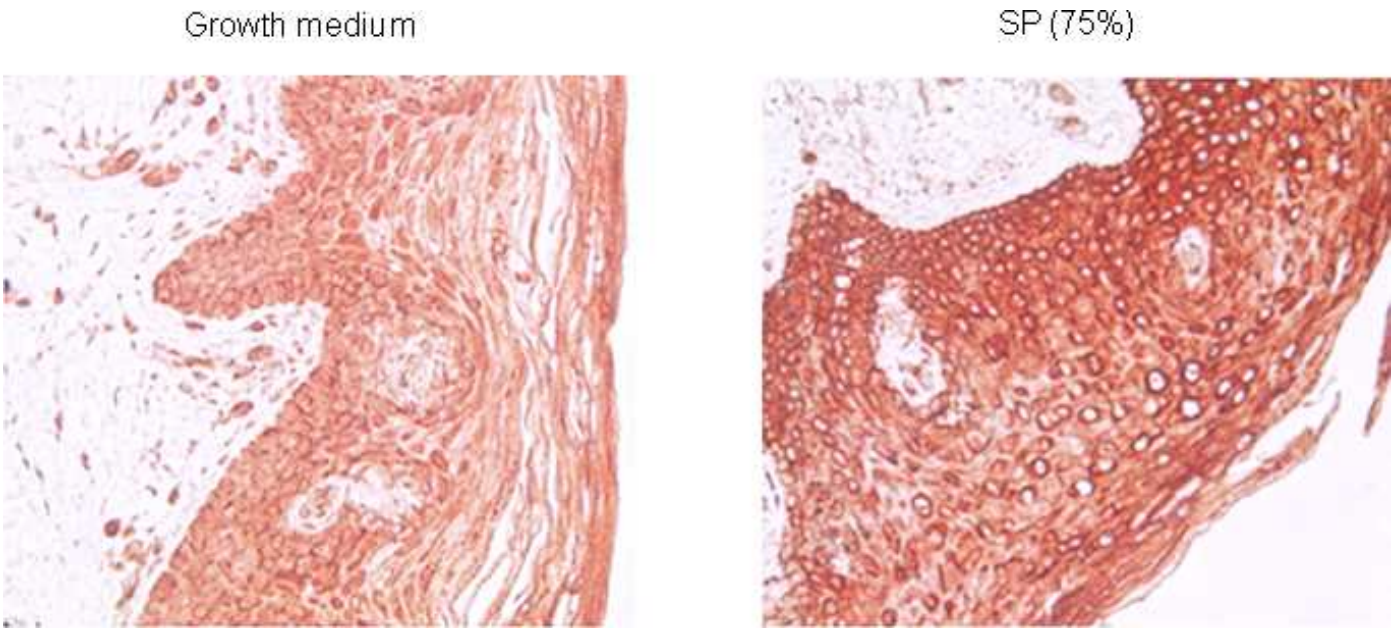

FIG. 9. SP induces PTGS2 in vaginal tissue explants in culture. Vaginal tissues were treated with SP (75%) for 24 h. PTGS2 expression was assayed by IHC. Original magnification ×400.

(Fig. 4). These data imply that, in vivo, SP could favor or increase inflammatory responses triggered by preexisting cevicovaginal infections. In addition, SP from men harboring genital infections could be more potent in eliciting inflammatory responses in the female genital mucosa than SP from healthy men. This is in agreement with reports of higher incidences of cervicitis in women whose partners are diagnosed with urethritis [38, 39]. Furthermore, our data suggest that exposure to SP in sexually active women may worsen mucosal inflammation caused by vaginal topical products, such as those containing N-9 (commercial spermicides) or IMQ (products to treat genital/anal warts) as active ingredients. Tested in a phase III clinical trial as an HIV microbicide, N-9 not only failed to protect women but actually increased susceptibility to HIV infection if used frequently [40]. Cervicovaginal mucosal inflammation induced by frequent exposure to N-9 has been postulated as a causative factor for the observed increased rate of HIV acquisition [41, 42]. Our data further suggest that frequent exposure to SP in this population of commercial sex workers could have exacerbated N-9-induced inflammatory response, thus increasing the propensity for HIV infection.

We have observed considerable variation in the PTGS2-inducing capacity of SP among different individuals (Fig. 5), which could be the result of interindividual variability in SP composition [43, 44]. We have also noted some variation among SP samples from the same individual. SP variability could be due to differences in genetic polymorphisms, lifestyle, sexual practices, and use of medications [45]. Other significant factors are subclinical infections and variations in the genital microbiome. Seasonal variations due to changes in daylight and temperature could in turn explain the differences in SP from the same person [45, 46].

Seminal plasma is a complex fluid that contains a multitude of biologically active molecules [43]. In our attempts to pinpoint the factor(s) responsible for PTGS2 stimulation, we found that small molecules, possibly of a nonprotein nature, might be plausible candidates (Fig. 6A). We hypothesized that these could be prostaglandins of the E-series, which are present in SP at uniquely high concentration (of at least three orders of magnitude higher than in other human fluids and tissues) [30]. PGE mediates its activity through four subtypes of G-protein-coupled receptors, EP1–EP4 [33]. We demonstrated that PTGS2 expression was abrogated in the presence of AH-6809, an antagonist of prostaglandin receptors that blocks EP1, EP2, and EP3. AH-6809 also suppresses receptor of PGD_2 [31]; however PGD_2 is not present in SP, which justifies the suggestion that the prostaglandin(s) involved in PTGS2 induction by SP belong to PGE family (Fig. 6C). One of the most important PGEs known to be involved in inflammation is PGE_2, a low-molecular-weight (352.5 Da) component present in SP. Importantly, being a product of PTGS2 activity, PGE_2 displays a feedback reaction by inducing PTGS2 expression in diverse cell types [21, 47]. This role of PGE_2 was confirmed by the direct effect of PGE_2 on PTGS2 stimulation in human vaginal cells when incubated with $dmPGE_2$ (Fig. 6D). At the same time, the role of other semen PGEs in PTGS2 induction cannot be excluded.

To further test the hypothesis of the role of PGE_2 in PTGS2 induction and find out whether there is a correlation between these two factors, we selected five SP samples with high and low PTGS2 response for quantitation of their PGE_2 content using liquid chromatography. We observed a positive correlation between PGE_2 levels and PTGS2 expression (Fig. 7B). Although more experiments are needed to confirm this correlation, an association between PGE_2 level in SP and SP ability to stimulate PTGS2 expression appears evident. PTGS2 upregulation in the genital tract induced by SP may result in an even higher level of PGE_2, which would further stimulate mucosal expression of PTGS2 [48].

Several studies have demonstrated a direct effect of PGE_2 on HIV-1 infectivity. PGE_2 promotes replication of the virus [49] and activates HIV-1 long terminal repeat (LTR)-mediated gene activity in T-cells [50]. Activation of HIV-LTR was also observed in the presence of SP [11]. This would point to a direct effect of SP in promoting HIV infection, separate from the reported facilitating effect of SP-derived amyloid fibrils [13, 51].

Furthermore, SP may also facilitate HIV infection indirectly. Involvement of PGE_2 in inflammation is well established. Acting via EP receptors, PGE_2 is capable of modulating the immune cells functions, and their activation might increase HIV-1 capture, transmission, and/or infection. A novel role of PGE_2 in immune activation as a factor that facilitates expansion of Th17 subset of T helper cells is now emerging [52]. Importantly, evidence is accumulating that Th17 cells are preferential targets for HIV-1/SIV [53, 54].

Recently, another molecule, transforming growth factor- β (TGF-β), which is present in extremely high concentration in human semen, has been shown to induce activation of a number of inflammation-related genes, including *PTGS2* in the female genital tract [55]. There are three isoforms of TGF-β in semen (TGF-β1, TGF-β2, TGF-β3); they exist predominantly in a latent form complexed with the latency-associated peptide (LAP). Physiological release from an inactive complex can be accomplished by proteolytic degradation of LAP. Physicochemical activation of TGF-β can be achieved by heat. Maximum activation of TGF-β2 and TGF-β3 occurs at 100°C, while TGF-β1 is denatured at this temperature[56]. Heat-activated TGF-β isoforms can account for PTGS2-inducing activity that we observed in vaginal cells treated with thermally treated SP (Fig. 6). It has been proposed that TGF-β can interact with PGEs to cause inflammation-related response [55].

It has been known for several decades that SP causes an inflammatory-like response in the female genital mucosa [18]. Increased migration of immune cells to the site of semen deposition, called leukocytic reaction [17, 57], and expression and release of diverse proinflammatory cytokines stimulated by SP have been observed in different mammals, including humans [58]. It has been proposed that these events facilitate conception [18]. While being physiologically beneficial for reproductive functions, the influx of immune cells that are targets for HIV infection may enhance HIV acquisition and transmission.

In conclusion, seminal plasma induces PTGS2 in vaginal epithelial cells through activation of NF-κB and MAPKs. SP-induced PTGS2 expression follows a specific and dose-dependent response and shows interindividual variability. Semen PGE_2 is involved in the PTGS2 induction. Increased mucosal expression of PTGS2, especially in the presence of microbial antigens and other proinflammatory stimuli, may have implications for HIV-1 transmission and the design of strategies to prevent sexually transmitted infections.

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REFERENCES

1. Cohen MS. HIV and sexually transmitted diseases: lethal synergy. Top HIV Med 2004; 12:104–107.

2. Schellenberg JJ, Plummer FA. The microbiological context of HIV resistance: vaginal microbiota and mucosal inflammation at the viral point of entry. Int J Inflam 2012; 2012:131243.
3. Thurman AR, Doncel GF. Innate immunity and inflammatory response to Trichomonas vaginalis and bacterial vaginosis: relationship to HIV acquisition. Am J Reprod Immunol 2011; 65:89–98.
4. Haase AT. Targeting early infection to prevent HIV-1 mucosal transmission. Nature 2010; 464:217–223.
5. Doncel GF, Joseph T, Thurman AR. Role of semen in HIV-1 transmission: inhibitor or facilitator? Am J Reprod Immunol 2010; 65:292–301.
6. Sabatte J, Lenicov FR, Cabrini M, Rodriguez CR, Ostrowski M, Ceballos A, Amigorena S, Geffner J. The role of semen in sexual transmission of HIV: beyond a carrier for virus particles. Microbes Infect 2011; 13: 977–982.
7. Martellini JA, Cole AL, Venkataraman N, Quinn GA, Svoboda P, Gangrade BK, Pohl J, Sorensen OE, Cole AM. Cationic polypeptides contribute to the anti-HIV-1 activity of human seminal plasma. FASEB J 2009; 23:3609–3618.
8. Sabatte J, Ceballos A, Raiden S, Vermeulen M, Nahmod K, Maggini J, Salamone G, Salomon H, Amigorena S, Geffner J. Human seminal plasma abrogates the capture and transmission of human immunodeficiency virus type 1 to CD4+ T cells mediated by DC-SIGN. J Virol 2007; 81: 13723–13734.
9. Klebanoff SJ, Kazazi F. Inactivation of human immunodeficiency virus type 1 by the amine oxidase-peroxidase system. J Clin Microbiol 1995; 33: 2054–2057.
10. Stief TW. Singlet oxygen (1O2)-oxidazable lipids in the HIV membrane, new targets for AIDS therapy? Med Hypotheses 2003; 60:575–577.
11. Kafka JK, Sheth PM, Nazli A, Osborne BJ, Kovacs C, Kaul R, Kaushic C. Endometrial epithelial cell response to semen from HIV-infected men during different stages of infection is distinct and can drive HIV-1-long terminal repeat. AIDS 2012; 26:27–36.
12. Gorodeski GI, Goldfarb J. Seminal fluid factor increases the resistance of the tight junctional complex of cultured human cervical epithelium CaSki cells. Fertil Steril 1998; 69:309–317.
13. Munch J, Rucker E, Standker L, Adermann K, Goffinet C, Schindler M, Wildum S, Chinnadurai R, Rajan D, Specht A, Gimenez-Gallego G, Sanchez PC, et al. Semen-derived amyloid fibrils drastically enhance HIV infection. Cell 2007; 131:1059–1071.
14. Berlier W, Cremel M, Hamzeh H, Levy R, Lucht F, Bourlet T, Pozzetto B, Delezay O. Seminal plasma promotes the attraction of Langerhans cells via the secretion of CCL20 by vaginal epithelial cells: involvement in the sexual transmission of HIV. Hum Reprod 2006; 21:1135–1142.
15. Ongradi J, Ceccherini-Nelli L, Pistello M, Specter S, Bendinelli M. Acid sensitivity of cell-free and cell-associated HIV-1: clinical implications. AIDS Res Hum Retroviruses 1990; 6:1433–1436.
16. 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.
17. Thompson LA, Barratt CL, Bolton AE, Cooke ID. The leukocytic reaction of the human uterine cervix. Am J Reprod Immunol 1992; 28:85–89.
18. Robertson SA. Seminal plasma and male factor signalling in the female reproductive tract. Cell Tissue Res 2005; 322:43–52.
19. Robertson SA, Guerin LR, Moldenhauer LM, Hayball JD. Activating T regulatory cells for tolerance in early pregnancy—the contribution of seminal fluid. J Reprod Immunol 2009; 83:109–116.
20. 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.
21. Sales KJ, Katz AA, Millar RP, Jabbour HN. Seminal plasma activates cyclooxygenase-2 and prostaglandin E2 receptor expression and signalling in cervical adenocarcinoma cells. Mol Hum Reprod 2002; 8:1065–1070.
22. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem 2000; 69:145–182.
23. Turini ME, DuBois RN. Cyclooxygenase-2: a therapeutic target. Annu Rev Med 2002; 53:35–57.
24. Tilley SL, Coffman TM, Koller BH. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. J Clin Invest 2001; 108:15–23.
25. Zalenskaya IA, Cerocchi OG, Joseph T, Donaghay MA, Schriver SD, Doncel GF. Increased PTGS2 expression in human vaginal epithelial cells exposed to nonoxynol-9, a vaginal contraceptive microbicide that failed to protect women from HIV-1 infection. Am J Reprod Immunol 2011; 65: 569–577.
26. Fichorova RN, Rheinwald JG, Anderson DJ. Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. Biol Reprod 1997; 57:847–855.
27. Zalenskaya IA, Bradbury EM, Zalensky AO. Chromatin structure of telomere domain in human sperm. Biochem Biophys Res Commun 2000; 279:213–218.
28. Chen CC. Signal transduction pathways of inflammatory gene expressions and therapeutic implications. Curr Pharm Des 2006; 12:3497–3508.
29. Joseph T, Zalenskaya IA, Yousefieh N, Schriver SD, Cote LC, Chandra N, Doncel GF. Induction of cyclooxygenase (COX)-2 in human vaginal epithelial cells in response to TLR ligands and TNF-alpha. Am J Reprod Immunol 67:482–490.
30. Templeton AA, Cooper I, Kelly RW. Prostaglandin concentrations in the semen of fertile men. J Reprod Fertil 1978; 52:147–150.
31. Abramovitz M, Adam M, Boie Y, Carriere M, Denis D, Godbout C, Lamontagne S, Rochette C, Sawyer N, Tremblay NM, Belley M, Gallant M, et al. The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. Biochim Biophys Acta 2000; 1483:285–293.
32. Cha YI, DuBois RN. NSAIDs and cancer prevention: targets downstream of PTGS2. Annu Rev Med 2007; 58:239–252.
33. Hata AN, Breyer RM. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol Ther 2004; 103:147–166.
34. Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 1998; 38:97–120.
35. 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.
36. Palm F, Walter I, Budik S, Kolodziejek J, Nowotny N, Aurich C. Influence of different semen extenders and seminal plasma on PMN migration and on expression of IL-1beta, IL-6, TNF-alpha and PTGS2 mRNA in the equine endometrium. Theriogenology 2008; 70:843–851.
37. Chun KS, Surh YJ. Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. Biochem Pharmacol 2004; 68:1089–1100.
38. Brunham RC, Paavonen J, Stevens CE, Kiviat N, Kuo CC, Critchlow CW, Holmes KK. Mucopurulent cervicitis—the ignored counterpart in women of urethritis in men. N Engl J Med 1984; 311:1–6.
39. Tait IA, Rees E, Hobson D, Byng RE, Tweedie MC. Chlamydial infection of the cervix in contacts of men with nongonococcal urethritis. Br J Vener Dis 1980; 56:37–45.
40. Van Damme L, Chandeying V, Ramjee G, Rees H, Sirivongrangson P, Laga M, Perriens J. Safety of multiple daily applications of COL-1492, a nonoxynol-9 vaginal gel, among female sex workers. COL-1492 Phase II Study Group. AIDS 2000; 14:85–88.
41. Fichorova RN, Tucker LD, Anderson DJ. The molecular basis of nonoxynol-9-induced vaginal inflammation and its possible relevance to human immunodeficiency virus type 1 transmission. J Infect Dis 2001; 184:418–428.
42. Doncel GF, Chandra N, Fichorova RN. Preclinical assessment of the proinflammatory potential of microbicide candidates. J Acquir Immune Defic Syndr 2004; 37(suppl 3):S174–S180.
43. Yamakawa K, Yoshida K, Nishikawa H, Kato T, Iwamoto T. Comparative analysis of interindividual variations in the seminal plasma proteome of fertile men with identification of potential markers for azoospermia in infertile patients. J Androl 2007; 28:858–865.
44. Owen DH, Katz DF. A review of the physical and chemical properties of human semen and the formulation of a semen simulant. J Androl 2005; 26: 459–469.
45. Politch JA, Tucker L, Bowman FP, Anderson DJ. Concentrations and significance of cytokines and other immunologic factors in semen of healthy fertile men. Hum Reprod 2007; 22:2928–2935.
46. Chen Z, Toth T, Godfrey-Bailey L, Mercedat N, Schiff I, Hauser R. Seasonal variation and age-related changes in human semen parameters. J Androl 2003; 24:226–231.
47. Maldve RE, Kim Y, Muga SJ, Fischer SM. Prostaglandin E(2) regulation of cyclooxygenase expression in keratinocytes is mediated via cyclic nucleotide-linked prostaglandin receptors. J Lipid Res 2000; 41:873–881.
48. Sales KJ, Jabbour HN. Cyclooxygenase enzymes and prostaglandins in reproductive tract physiology and pathology. Prostaglandins Other Lipid Mediat 2003; 71:97–117.
49. Kuno S, Ueno R, Hayaishi O, Nakashima H, Harada S, Yamamoto N. Prostaglandin E2, a seminal constituent, facilitates the replication of acquired immune deficiency syndrome virus in vitro. Proc Natl Acad Sci U S A 1986; 83:3487–3490.

50. Dumais N, Barbeau B, Olivier M, Tremblay MJ. Prostaglandin E2 up-regulates HIV-1 long terminal repeat-driven gene activity in T cells via NF-kappaB-dependent and -independent signaling pathways. J Biol Chem 1998; 273:27306–27314.
51. Kim KA, Yolamanova M, Zirafi O, Roan NR, Staendker L, Forssmann WG, Burgener A, Dejucq-Rainsford N, Hahn BH, Shaw GM, Greene WC, Kirchhoff F, et al. Semen-mediated enhancement of HIV infection is donor-dependent and correlates with the levels of SEVI. Retrovirology 2010; 7:1742–4690.
52. Sakata D, Yao C, Narumiya S. Prostaglandin E2, an immunoactivator. J Pharmacol Sci 2010; 112:1–5.
53. El Hed A, Khaitan A, Kozhaya L, Manel N, Daskalakis D, Borkowsky W, Valentine F, Littman DR, Unutmaz D. Susceptibility of human Th17 cells to human immunodeficiency virus and their perturbation during infection. J Infect Dis 2010; 201:843–854.
54. Khader SA, Gaffen SL, Kolls JK. Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. Mucosal Immunol 2009; 2:403–411.
55. 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.
56. Brown PD, Wakefield LM, Levinson AD, Sporn MB. Physicochemical activation of recombinant latent transforming growth factor-beta's 1, 2, and 3. Growth Factors 1990; 3:35–43.
57. Pandya IJ, Cohen J. The leukocytic reaction of the human uterine cervix to spermatozoa. Fertil Steril 1985; 43:417–421.
58. Robertson SA. Seminal fluid signaling in the female reproductive tract: lessons from rodents and pigs. J Anim Sci 2007; 85:E36–E44.