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Interferon epsilon in the reproductive tract of healthy and genital herpes simplex virus-infected pregnant women: Results of a pilot study

Colette Nickodem[1] | Michael F. Criscitiello[2,3] | Fuller Bazer[4] | Olayinka Abiodun-Ojo[1] | Brandie D. Taylor[1]

[1]Department of Epidemiology and Biostatistics, Texas A&M University, College Station, TX, USA

[2]Comparative Immunogenetics Laboratory, Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, USA

[3]Department of Microbial Pathogenesis and Immunology, College of Medicine, Texas A&M University, College Station, TX, USA

[4]Department of Animal Science, Texas A&M University, College Station, TX, USA

Correspondence
Brandie DePaoli Taylor, Department of Epidemiology and Biostatistics, Texas A&M University, College Station, TX, USA.
Email: taylor@sph.tamhsc.edu

Problem: Recently characterized interferon epsilon (IFNe) protects against sexually transmitted infections, including genital herpes simplex virus (HSV), in animal models. There are no reports of IFNe in genital tract secretions of pregnant women, and data on IFNe in non-pregnant women are limited. This pilot study is the first to measure concentrations of IFNe in vaginal and cervical secretions during pregnancy and compare values between healthy and genital HSV-infected women.

Method of Study: Vaginal or cervical specimens from 30 pregnant women were obtained from the Global Alliance to Prevent Prematurity and Stillbirth (GAPPS) repository. Cervical samples were collected during the first trimester and vaginal samples across pregnancy. Enzyme-linked immunosorbent assay determined concentrations of IFNe (pg/mL). Data for IFNe were log-transformed and compared by maternal demographics, clinical variables, and HSV status using t tests and linear regression. Repeated measures analysis explored trends across pregnancy.

Results: Among the entire cohort, first trimester concentrations of IFNe in vaginal or cervical secretions decreased as body mass index increased ($\beta = -0.14$, $P = .0466$). Concentrations of vaginal IFNe increased across pregnancy in HSV-infected and healthy women ($P = .009$). Average vaginal IFNe across pregnancy was lower in women with HSV compared to healthy women ($P = .0009$).

Conclusion: Interferon epsilon increased across pregnancy, but was less abundant in women with HSV. This pilot investigation cannot make any definitive conclusions. However, animal models suggest that IFNe may protect against STIs. Thus, larger studies are required to validate expression of IFNe in the reproductive tract of pregnant women with and without genital infections.

KEYWORDS
cytokines, pregnancy, sexually transmitted infection

1 | INTRODUCTION

Studies of interferon epsilon (IFNe) in humans are needed as this unique type 1 IFN is constitutively expressed in the female reproductive tract and protects against sexually transmitted infections (STIs) in animal models.[1-3] Mice deficient in IFNe have increased susceptibility to viral sexually transmitted infections (STIs) including genital herpes simplex virus (HSV), *Chlamydia trachomatis*, and HIV.[1,4] For example, inoculation with high doses of HSV into the genital tract of mice with greater concentrations of IFNe in the

vagina results in less severe disease compared to mice with low concentrations of IFNe. These novel findings have led to a great interest in IFNe as a novel therapeutic for STIs. However, human studies of IFNe are rare, and it is not known whether IFNe has clinical significance.

Most type I IFNs are activated by Toll-like receptors (TLR) following recognition of pathogen-associated molecular patterns.[5] Excessive TLR signaling may exacerbate type I IFN-associated disease.[6,7] However, IFNe is unique to epithelial cells of the female reproductive tract, and its expression is regulated by estrogen and progesterone.[1-3] Indeed, expression of IFNe is upregulated in mice after administration of estrogen.[1] Similarly, the abundance of IFNe in epithelial cells from human uterine endometrium (n = 6) is 10-fold lower during the secretory phase (dominated by progesterone) compared to the proliferative phase (dominated by estrogen) of the menstrual cycle.[1] The secretory phase is associated with immunological changes that support embryonic development and implantation,[8,9] but increased vulnerability to pathogens.[10,11] As expression of IFNe is not induced via TLRs, it may be a more desirable mucosal therapeutic or vaccine target for STIs.[12]

Viral infections of the genital tract are a significant public health concern, particularly for women of reproductive age. HSV-2 is one of the most common STIs in the United States, disproportionately affecting women.[13,14] It is estimated that genital HSV affects 50 million American adults, but this is likely underestimated as the virus is asymptomatic in 65%-90% of cases.[13,15] Approximately one in five pregnant women are seropositive for genital HSV-2. A recent study of 422 pregnant Brazilian women revealed that the placental prevalence of HSV-2 and HSV-1 was 12.6% and 28.0% (maternal side) and 8.3% and 29.9% (fetal side).[16] Transmission from mother to offspring can lead to significant morbidities, including aseptic meningitis and death in 23%-29% of cases of neonatal herpes.[17-19]

Type one IFNs have immunomodulatory roles during pregnancy and promote tolerance to the conceptus (fetus and placenta) and protect against infection.[20] Prenatal viral infections are hypothesized to alter type I IFN responses to bacteria (commensal or pathogenic) which may lead to adverse pregnancy outcomes.[11] While attention has been directed at possible roles of IFNe in protection against genital infections in non-pregnant animal models, studies have not considered if this type I IFN may be an important immune modulator during pregnancy. To our knowledge, there are no reports of the presence of IFNe in the reproductive tract of women or other mammals during various stages of pregnancy. Furthermore, the only data from non-pregnant women are from six endometrial samples where expression of IFNe mRNA was measured by QT-PCR as part of a mouse model study.[1] The objective of this pilot study was to determine whether we could detect IFNe in vaginal and cervical secretions. The severity of genital HSV decreases in mice with greater amounts of IFNe in the vagina.[1] Therefore, our secondary objective was to determine abundances of IFNe in genital tract secretions of women with healthy pregnancies compared to pregnant women with HSV across the trimesters of pregnancy.

2 | MATERIALS AND METHODS

2.1 | Sample collection

This pilot study included a random sample of 30 women from the Global Alliance to Prevent Prematurity and Stillbirth (GAPPS) at the Seattle Children's Hospital. Women were eligible for selection if they had singleton pregnancies, delivered at term (≥37 weeks of gestation) and did not have preexisting chronic diseases. Further, women had to have first trimester cervical swabs or vaginal swabs from all three trimesters available for analysis. The GAPPS repository is a biobank that collects specimens from women beginning at the first prenatal visit and then longitudinally across pregnancy and to 6 weeks postpartum. Women obtained prenatal care from the University of Washington Medical Center, Yakima Valley Memorial Hospital or the Swedish Medical Center. All women provided informed consent. GAPPS is approved by the Seattle Children's Institutional Review Board.

As IFNe had not been measured in genital tract secretions previously, we aimed to determine whether IFNe was detectable in vaginal and cervical swabs. We obtained a single cervical swab from 15 healthy pregnant women during the first trimester of pregnancy which is the only time point GAPPS collects cervical samples (gestational age range: 8-12 weeks). We obtained vaginal swabs taken during the first (range 8-12 weeks), second (range 16-25 weeks), and third (range 28-37 weeks) trimesters of pregnancy from 10 healthy women and from 5 women with genital HSV. All 15 of these women had vaginal swabs collected at each trimester of pregnancy (total of 45 swabs). We were unable to obtain cervical and vaginal swabs from the same individuals. The Texas A&M University institutional review board approved the current investigation.

Global Alliance to Prevent Prematurity and Stillbirth uses a web-based data management system (LabVantage) to store and track data for participants (demographic, consent, and delivery-related data) and specimens (collection time, storage data, and test results). As routine serological screening for HSV is not recommended during pregnancy,[21,22] women who were documented as HSV positive in the GAPPS database were symptomatic (evidence of herpetic lesions). Suspected cases may receive a confirmatory viral DNA polymerase chain reaction test or serological test per ACOG and CDC guidelines.[21,22] Women are treated orally each day with either acyclovir (400 mg) or valacyclovir (1 g). We obtained additional data on age, self-reported race/ethnicity, body mass index (BMI), medications used during pregnancy, gravidity, fetal sex, and gestational age at delivery. We obtained data on infections and confirmed that women with HSV did not have coinfections.

2.2 | Sample procedures

All biological samples in GAPPS are collected by trained staff using standardized collection kits and protocols during collection,

TABLE 1 Concentrations of interferon epsilon in cervical and vaginal swabs by trimester

Time and sample type	Raw mean (pg/mL) (SD)	Raw median (pg/mL) (IQR)	Proportion below limit of detection
First trimester cervical	54.58 (54.62)	35.50 (99.54)	0.267
First trimester vaginal	186.76 (403.59)	12.24 (58.89)	0.333
Second trimester vaginal	457.80 (386.42)	415.00 (653.66)	0
Third trimester vaginal	485.92 (468.89)	209.09 (603.05)	0

SD, standard deviation; IQR, interquartile range.

storage, and distribution. GAPPS maintains adherence to the International Society for Biological and Environmental Repositories (ISBER) best practices for repositories[23] as well as implements numerous in-house programs to monitor and maintain specimens and data quality. Lubricant is avoided during collection of specimens. After speculum insertion, the cervix is cleaned of mucus and posterior fornix specimens are collected using polyester-tipped swabs (Fisher Scientific). Vaginal midpoint specimens are collected using sterile polyester-tipped swabs scraped across the vaginal sidewall about halfway between the introitus and the cervix. All swabs are air-dried prior to placement in collection tubes with 1 mL of sterile phosphate-buffered saline solution (PBS) and arranged in a microcentrifuge rack within 15 minutes. Tubes are centrifuged for 10 seconds to ensure that material is evacuated into the stabilizing liquid. Swabs are placed in 2-mL cryovial tubes and plastic shafts are snapped off. Tubes are recapped and put on dry ice or directly into the −80°C freezer within 5 minutes. All samples are frozen within 4 hours. These standard operating procedures are commonly used when processing female genital tract specimens for cytokine detection.[24,25]

Cervical and vaginal swabs (with no recorded thaws) were shipped to Texas A&M University for determination of concentrations of IFNe (pg/mL) using an enzyme-linked immunosorbent assay (ELISA). Samples were thawed from −80°C for 10 minutes before rocking at 4°C for 1 hour in 300 microliters of PBS. Swab tips were squeezed against the side of tube to elute the sample and the solution was centrifuged for 10 minutes at 14 000× gravity in a microcentrifuge to pellet fibers. Supernatants were stored at 4°C overnight and assayed the following day. This elution procedure is common for recovery of genital tract secretions.[25,26]

2.3 | IFNe enzyme-linked immunosorbent assay (ELISA)

Samples were analyzed in duplicate for IFNe using a quantitative sandwich ELISA according to the manufacturer's instructions (MyBiosource, Vancouver). Intra-assay precision was calculated by the manufacturer to be CV% <8%, interassay precision to be <10%, and the assay has a range of detection of 15.6-1000 pg/mL. The r^2 value of our standard curve was 0.9962. No significant cross-reactivity has been found by the MyBiosource with any IFN homolog.

Briefly, antibody specific for IFNE was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any IFNE present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for IFNE was added to the wells. After washing, avidin-conjugated horseradish peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and color was allowed to develop in proportion to the amount of IFNE bound in the initial step. The color development was stopped after exactly 20 minutes at 37 degrees centigrade and the intensity of the color measured at an optical density of 450 nm.

2.4 | Statistical analysis

Our primary objective was to determine whether IFNe was detectable in genital tract specimens. Once IFNe was detected in cervical and vaginal specimens, we examined the distribution of IFNe for skewness and statistical tests of normality (eg, Kolmogorov-Smirnov test). IFNe was not normally distributed in cervical or vaginal samples. Subsequently, IFNe (pg/mL) was log-transformed prior to analyses. We compared the geometric means of IFNe between specimen types to determine whether we could combine results to make comparisons by demographic and clinical variables among all 30 women to increase our power. There was no difference in the distribution of IFNe at 8-12 weeks gestation between specimen types [geometric mean 3.95 (SD 1.7) vs 3.92 (SD 1.1) $P = .977$], and data were combined to compare IFNe levels by demographic and clinical variables. To compare IFNe geometric means, independent t tests were used for binary demographic and clinical variables and linear regression for continuous variables.

Our secondary objective was to examine the abundance of IFNe in genital tract secretions across pregnancy in healthy and HSV-infected women. Only vaginal swabs were collected across pregnancy. The independent *t* test was used to compare IFNe by HSV status. This was done using the average of IFNe across pregnancy, as well as examining concentrations at each trimester. We then examined trends in IFNe levels across each time point of pregnancy. Generalized linear models were used for repeated measures of variance analyses to evaluate changes in detectable IFNe across each pregnancy time point in HSV and healthy women. An interaction between time (time point of sample collection) and outcome status (HSV yes/no) was included to determine whether trends across pregnancy were similar in both healthy and HSV-infected women. SAS version 9.4 (Cary, NC) was used for analyses.

TABLE 2 Distribution of sample demographic and clinical characteristics of the 30 women included in the study and comparisons of IFNe levels measured at 8- to 12-week gestation

Characteristics	[a]Overall Distribution of characteristics among the cohort	Comparison of IFNe by characteristics	
		[b]β1 (SE) or geometric means (SD)	[b]*P*-value
Age	29 (7)	β = −0.002 (0.06)	.9675
BMI	25.19 (4.06)	β = −0.14 (0.07)	.0466
Gestation age delivery	39.57 (1.43)	β = 0.43 (0.29)	.1544
White race	26 (0.87)	3.88 (1.3)	.7183
Non-white race	4 (0.13)	4.19 (2.3)	
First pregnancy	18 (0.6)	4.5 (2.5)	.3664
2+ pregnancies	12 (0.4)	3.5 (0.67)	
Female fetal sex	14 (0.47)	4.0 (1.7)	.8619
Male fetal sex	16 (0.53)	3.8 (2.2)	

SE, standard error; SD, standard deviation.
[a]Distributions are presented for continuous independent variables (age, BMI, and gestational age at delivery) as median (IQR; interquartile range). All other categorical independent variables are presented as frequency (percent).
[b]IFNe levels were compared by continuous independent variables using linear regression and for all other independent t tests were used.

TABLE 3 Concentrations of IFNe in vaginal swabs related to HSV status

	Average (pg/mL)	First trimester (pg/mL)	Second trimester (pg/mL)	Third trimester (pg/mL)
[a]No HSV, geometric mean (SD)	6.1 (0.6)	4.5 (2.1)	6.0 (1.0)	6.1 (1.1)
HSV, geometric mean (SD)	4.5 (0.9)	3.1 (1.2)	4.8 (1.1)	4.4 (1.2)
P-value	.0009	.3136	.0415	.0158

SD, standard deviation.
[a]Analyzed using an independent *t* test.

3 | RESULTS

Overall, 26.7% of the first trimester cervical swabs had undetectable IFNe and 33.3% of the first trimester vaginal swabs had undetectable IFNe (Table 1). IFNe was detectable in all vaginal samples from the second and third trimesters.

Among the 30 participants, women were primarily white (83.3%), younger than 30 years of age (53.3%) and had a body mass index (BMI) ≤25 kg/m^2 (53.3%) (Table 1). Cervical or vaginal IFNe (measured 8-12 weeks of gestation) decreased with each unit decrease in BMI (β = −0.14, *P* = .0466; Table 2. There was no relationship between maternal age (β = −0.002, *P* = .9675) or gestational age of delivery (β = 0.43, *P* = .1544) and concentrations of IFNe. Concentrations of IFNe were not different by race [geometric mean 3.88(SD 1.3) vs 4.19(SD 2.3); *P* = .7183], gravidity (first pregnancy vs two or more pregnancies) [4.5(2.5) vs 3.5(0.67); *P* = .3664] or fetal sex [4.0(1.7) vs 3.8(2.2); *P* = .8619]. Results were similar when cervical and vaginal samples were examined separately.

Table 3 summarizes concentrations of IFNe in vaginal samples by HSV status for each trimester of pregnancy. Average concentration of IFNe across pregnancy was less for women with HSV infection [geometric mean 4.5 (0.9) vs 6.1 (0.6); *P* = .0009]. When examined by trimester, the relationship was only statistically significant for samples collected during the second and third trimesters (*P* = .0415 and *P* = .0158, respectively).

Herpes simplex virus-infected and healthy women had increasing concentrations of IFNe in vaginal samples from the first to third trimester of pregnancy (*P* = .009; Figure 1). However, the time of collection by HSV status interaction was not significant (*P* = .8872), which suggests that trends in IFNe across pregnancy were the same between healthy and HSV-infected women (ie, both displayed increasing trends at each time point of collection).

4 | DISCUSSION

This is the first study to determine whether IFNe is expressed in the human genital tract during pregnancy. IFNe was detected in vaginal and cervical swabs during the first trimester of pregnancy, although only 33.3% and 26.7% of the samples had detectable levels, respectively.

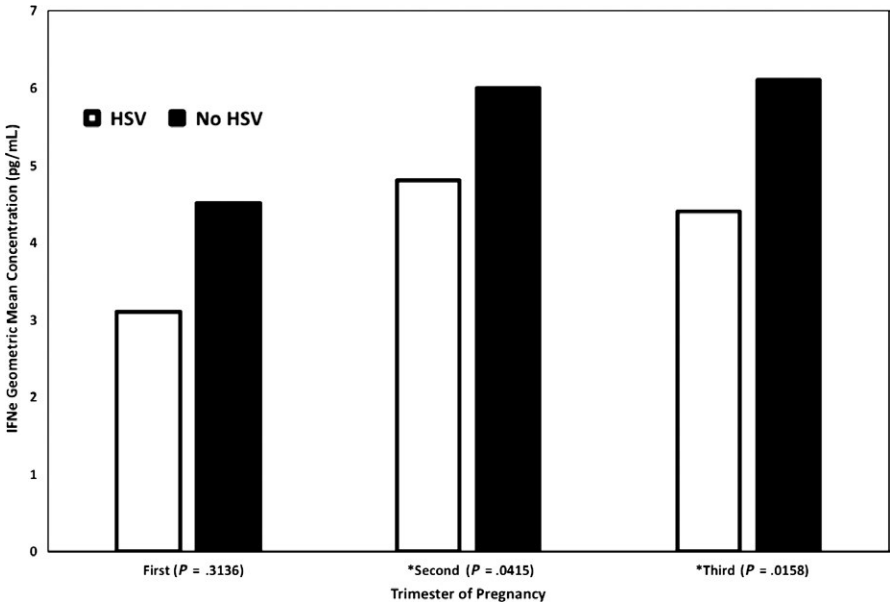

FIGURE 1 Figure 1 shows the vaginal concentrations(pg/mL) of IFNe in the first, second, and third trimesters of pregnancy in HSV infected women (shown in white) compared with healthy women (shown in black). There was a significant average difference in IFNe between healthy and HSV infected women ($P = .0009$). However, the difference was only significant in the second ($P = .0415$) and third trimesters ($P = .0158$)

We were only able to obtain vaginal swabs from women in each trimester of pregnancy and all women had detectable levels of IFNe in the second and third trimesters. IFNe increased from early to late stages of pregnancy in healthy and in HSV-infected women. However, due to the limitations of our pilot study we cannot make definitive conclusions regarding IFNe levels among HSV-infected women, these results need to be validated in a study with a larger cohort of women.

Interferon epsilon was lower in individuals with genital HSV compared to healthy women at multiple time points across pregnancy, but only statistically significant in the second and third trimesters, as well as for concentrations of IFNe when averaged for healthy vs genital HSV-infected women across all stages of gestation. The sample size of this pilot study reduced the power to detect smaller differences among groups and limits conclusions we can make regarding IFNe levels in HSV-infected women. Fung et al. have shown that higher levels of genital IFNe protect against genital HSV acquisition after administration of low doses to induce infection in a mouse model.[1] Higher levels of IFNe are correlated with lower clinical sores, epidermal lesions, and viral titers.[1] The mechanisms are of protection are not entirely clear. Human IFNe upregulates HIV restriction factors in multiple cell lines.[4] Viral replication may be restricted through the upregulation of Tripartite motif-containing protein 5 alpha (TRIM5α) and Interferon-induced GTP-binding protein Mx2 expression in phytohaemagglutinin-activated peripheral blood lymphocytes.[2] Hermant et al., have shown that IFNe is not induced after viral infection in multiple cell lines, suggesting that IFNe requires a cofactor for expression.[3] With respect to the present study, we cannot establish a temporal relationship between concentrations of IFNe and HSV due to a lack of data on exact timing of diagnosis of HSV. Although all HSV-infected women were symptomatic, we had no data on clearance or severity of infection.

Concentrations of IFNe in cervical or vaginal specimens were lower with increasing pre-pregnancy BMI in the first trimester. There is an association between BMI and increases in circulating levels of cytokines during pregnancy that can disrupt actions of reproductive hormones.[27,28] However, as human studies are limited, it is unknown if maternal characteristics influence expression of IFNe in the genital tract. Type 1 IFN-tau (ruminants) is the pregnancy recognition signal required for establishing and maintaining pregnancy.[29,30] Animal models have shown that IFN-tau has anti-inflammatory effects that may be beneficial for treating obesity, diabetes, and other inflammatory conditions.[30,31] This begs the question of whether IFNe, which has similar properties to IFN-tau, may reduce inflammation in the reproductive tract. Because of our limited sample size and lack of variability in demographic and clinical characteristics in the cohort, we were unable to fully examine differences in IFNe by these variables. Further investigations should explore the impact of maternal factors on expression of IFNe during pregnancy.

Our study is limited by the inclusion of relatively healthy women, without complications, who delivered at term. Thus, we cannot make inferences on the role(s) of IFNe regarding reproductive health and pregnancy as results of our study are not generalizable. Future investigations should further explore the role(s) of IFNe during pregnancy and its relationship to adverse outcomes, such as infection and inflammation that impact pregnancy. Immune molecules (eg., cytokines and lymphokines) are tightly involved in successful implantation, placentation, fetal growth, and parturition.[20] Type one IFNs have immunomodulatory roles during pregnancy, promote tolerance to the fetus, and protect against infection.[32,33] However, viral or bacterial insults can alter functions of IFNs,[34] leading to excessive inflammation or reduced receptivity to implantation/placentation during pregnancy. Furthermore, altered immune responses within the vagina may increase susceptibility to viruses and microbes or increase the risk of ascension of pathogens into the upper genital tract during pregnancy. For example, infection-induced inflammation is estimated to account for up to 20%-40% of preterm births,[35] and several studies have found increases in vaginal immune biomarkers to be associated with preterm birth.[36-38] Our own work has shown that vaginal cytokines

are more abundant in women with spontaneous preterm delivery due to histochorioamniotitis, which suggests an increased inflammatory response following microbial infection of the genital tract.[39] Future work should explore if altered IFNe levels during pregnancy lead to infection-induced adverse pregnancy outcomes.

Although this pilot study included a small number of participants, the results revealed that IFNe is detectable in vaginal secretions during all stages of pregnancy. We found a trend for an increase in the abundance of IFNe in vaginal secretions from the first through third trimesters of gestation. IFNe levels were lower in women infected with HSV in the second and third trimesters. While trends were similar for the first trimester, they were not statistically significant. This may be due to our reduced power (post hoc power for *t* test was 34%) which may have also affected comparisons of IFNe by maternal demographics and clinical characteristics. Because IFNe had not been measured in pregnant women previously, we used the total eluted sample for analysis. Thus, we were unable to account for total protein level. Future work is required to validate amounts of IFNe in vaginal secretions across pregnancy in healthy women and women with vaginal infections. Large longitudinal studies prior to and across stages of pregnancy can determine if IFNe protects against infection or impacts maternal and infant health. Animal models suggest that IFNe may be a novel therapeutic target against STIs. However, only after additional human studies involving non-pregnant and pregnant women are conducted can researchers determine if IFNe has clinical utility. This would be significant as STIs, reproductive health and successful outcomes of pregnancy are major public health concerns.

ORCID

Colette Nickodem http://orcid.org/0000-0002-3064-5833
Brandie D. Taylor http://orcid.org/0000-0002-8234-1815

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