UAGro
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Facultad de Ciencias Químico Biológicas
Unidad Académica de Medicina
Unidad de Investigación Especializada en Microbiología

Maestría en Ciencias Biomédicas

"Efecto de la infección por *Gardnerella vaginalis* sobre la respuesta inmune innata en lesión escamosa intraepitelial de cérvix uterino y la línea celular HeLa"

TESIS

QUE PARA OBTENER EL GRADO DE:

MAESTRÍA EN CIENCIAS BIOMÉDICAS

P R E S E N T A:

Q.B.P. Miying Dessire Gómez Cervantes

Directora de tesis: **Dra. Amalia Vences Velázquez**

Codirectora: **Dra. Luz del Carmen Alarcón Romero**

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UNIVERSIDAD AUTÓNOMA DE GUERRERO
FACULTAD DE CIENCIAS QUÍMICO BIOLÓGICAS
FACULTAD DE MEDICINA
UNIDAD DE INVESTIGACIÓN ESPECIALIZADA EN MICROBIOLOGÍA
MAESTRÍA EN CIENCIAS BIOMÉDICAS

APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 18 días del mes de junio de dos mil dieciocho se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado de la Maestría en Ciencias Biomédicas, para examinar la tesis titulada **"Efecto de la infección por *Gardnerella vaginalis* sobre la respuesta inmune innata en lesión escamosa intraepitelial de cérvix uterino y la linea celular HeLa"**, presentada por la alumna Miying Dessire Gómez Cervantes, para obtener el Grado de Maestría en Ciencias Biomédicas. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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Este trabajo fue realizado en el Laboratorio de Inmunidad Innata en la Interacción Hospedero-Patógeno del Centro Multidisciplinario de Estudios en Biotecnología-FMVZ, de la Universidad Michoacana de San Nicolás de Hidalgo, en el Laboratorio de Investigación de Inmunobiología y Diagnóstico Molecular y en el Laboratorio de Investigación de Citopatología e Inmunohistoquímica de la Facultad de Ciencias Químico Biológicas, de la Universidad Autónoma de Guerrero.

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Durante el periodo en que se cursó la Maestría en Ciencias Biomédicas, la Q.B.P. Miying Dessire Gómez Cervantes, recibió beca CONACYT con No. De registro: 705003.

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Keywords:	Bacterial vaginosis, Gardnerella vaginalis, innate immune response, inflammation

Dear Dr. Goméz-Cervantes:

Your manuscript entitled "Gardnerella vaginalis modulates the innate immune response in HeLa cell line evoking local inflammation" by Goméz-Cervantes, Miying; Cortés-Sarabia, Karen; López-Meza, Joel E.; Ochoa-Zarzosa, Alejandra; Guzmán-Guzmán, Iris; Parra-Rojas, Isela; Vences-Velázquez, Amalia, has been successfully submitted online and is presently being given full consideration for publication in Microbiology and Immunology.

Your manuscript ID is MAI-2018-203

Thank you for your participation.

Sincerely,
Microbiology and Immunology Editorial Office.

Gardnerella vaginalis modulates the innate immune response in HeLa cell line evoking local inflammation.

Running title: *Gardnerella vaginalis* regulates innate immunity

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ABSTRACT

Bacterial vaginosis (BV) is a polymicrobial clinical entity characterized by the lack of inflammatory cells. However, in cervicovaginal fluids of women with BV, alterations in inflammatory cytokine levels have been reported which has not been directly attributed to infection by *Gardnerella vaginalis* (main etiological agent). To which, the aim of this study was investigated the role of *G. vaginalis* in the innate immune response modulation. Hela cells were infected with *G. vaginalis* ATCC 14018 with a Multiplicity of infection (MOI) 1 and 20 for 2 and 4 h. Cell viability was analyzed by trypan blue exclusion assays, also inflammatory cytokines concentration, Toll-like receptor-4 (TLR4) membrane abundance and apoptosis induction was assessed by flow cytometry. We observed that *G. vaginalis* infection induced apoptosis (2.6-folds) in the 42% of the infected cells, increased inflammatory cytokines levels (IL-1β, IL-6, IL-10, TNF-α and IL-12p70) and TLR4 membrane abundance (2-folds). Our study provides evidence about the innate immune response of HeLa cells against *G. vaginalis* infection under low and high MOI, which could provide new insight for the role of this bacterium modeling the local inflammation.

Keywords: Bacterial vaginosis, *Gardnerella vaginalis*, innate immune response, inflammation.

INTRODUCTION

Bacterial vaginosis (BV) is a clinical entity characterized by the replacement of the normal microbiota composed by lactobacilli, as a consequence of facultative anaerobic microorganism's overgrowth (1). The most affected group are reproductive aged women, which has been associated with gynecological and obstetric diseases such as endometritis and premature births (2,3). Several etiological agents are related with this condition, among them: *Prevotella spp*., *Bacteroides spp*., *Peptostreptococcus spp*., *Atopobium vaginae*., *Mobiluncus spp*., and *Gardnerella vaginalis,* the latter considered the main etiological agent, since it's isolated in up to 90% of cases (4).

G. vaginalis is facultative anaerobic, small Gram-variable rods from 0.5 to 1.5 μm length, does not possess flagella, endospores or typical capsules, but it has fimbriae that cover the cell surface (5,6). This bacterium is classified into 8 different biotypes and at least 4 genotypes (7,8). Present a high pathogenic potential, due to the large number of virulence factors: 1) biofilm formation that confers resistance to the host immune response and treatment to antibiotics (9); 2) vaginolysin (VLY) production, a protein that lyses erythrocyte and epithelial cells (10); 3) the production of substances that modulate the immune response, such as succinate, that inhibits neutrophils and monocytes chemotaxis (11,12), and enzymes such as prolidase, carboxylase and sialidase, that degrade the cervical mucus promoting micro-abrasions increasing the susceptibility to acquire infections by other microorganisms (13-16).

At immunological level, BV is characterized by the lack of cells from the inflammatory response, despite the fact that the genital tract is massively colonized (17). Previous reports

have shown high levels of proinflammatory cytokines such as IL-1α, IL-1β, IL-6 and a decrease of IL-8 in the cervicovaginal fluids of women with BV (18). Additionally, it has been reported that *G. vaginalis* induces a significant increase in the secretion of proinflammatory cytokines (IL-1β, IL-18, and TNF-α) and cellular death (19). Inflammatory cytokine secretion is regulated by signals through the recognition of bacterial ligands by Toll-like receptors (TLRs). The TLRs are present in several types of cells in the genital tract, where they recognize a wide variety of bacterial compounds that include lipopolysaccharide (LPS), peptidoglycan, heat shock proteins (HSP), etc., evoking an inflammatory response for microorganism's elimination (20**).**

On the other hand, although BV is typically distinguished by the scare presence of cells in the inflammatory response, an increased level of pro and anti-inflammatory proteins as well as TLRs expression have been found in the cervicovaginal fluids of women with BV, that suggest an active innate immune response against the abnormal microbial colonization. However, it has not been shown that observed changes are specifically modulated by *G. vaginalis*, which was the main objective of this work.

MATERIALS AND METHODS

Gardnerella vaginalis culture and growth kinetics

Gardnerella vaginalis ATCC 14018 was cultured anaerobically at 37 °C with 5% of CO_2 for 24 h in Trypticasein Soy Agar (TSA) (Becton, Dickinson and Company, Sparks MD, USA). To establish the growth kinetics of *G. vaginalis*, a bacterial colony was inoculated into 5 mL of thioglycollate and brain heart infusion broth (BHI) (Becton, Dickinson and Company, Sparks MD, USA), and incubated at 37 °C with 5% of CO_2 at 180 rpm, overnight. Further,

optical density (OD), was adjusted to 0.2 (equivalent to 15 x 10^6 CFU/mL) and finally, 5 mL of both broths were inoculated with 1 mL. Bacterial growth was monitored at 1, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h. Lectures were measured at 595 nm in a spectrophotometer (Bio Rad iMark). Broth without inoculation was used as negative control.

HeLa cell culture

HeLa cells (derived from a woman with cervical adenocarcinoma and HPV 18 infection) were grown on Petri dishes (Corning-Costar) in growth medium that was mixture of DMEM medium/F-12 (DMEM/F-12K) (Sigma) supplemented with 10% Fetal Bovine Serum (FBS, Sigma), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco) and 1 µg/mL amphotericin B (Invitrogen). HeLa cells were grown in 5% CO_2 atmosphere at 37 °C, before infection with *G. vaginalis*, cells were cultured in serum-free medium without antibiotics for 24 h.

HeLa cells viability post-infection with *Gardnerella vaginalis*

Cellular viability was tested using the trypan blue exclusion assay. Briefly, 30,000 cells were incubated in DMEM medium (DMEM/F12K, Sigma) without FBS and antibiotics for 24 h at 37 °C in 24-well plates (Corning-Costar). The cells were infected during 2, 4 and 6 h, with the following multiplicities of infection (MOI); 1:1 and 20:1 bacterium per cell (15 x 10^6 CFU/mL) in 5 % of CO_2 at 37 °C. Then the cells were harvested by trypsinization and 10 µL of the cell suspension was mixed with 10 µL of trypan blue (0.4 %). Dead and viable cells were counted using a hematocytometer in an inverted microscope (Primo Vert, Zeiss). Cells were counted using the following formula: $\frac{cell\ counting}{5} = w \times 10{,}000 = X \times dilution\ factor = y \times 2$, considering cells without infection as the 100% of viability.

Apoptosis

The apoptosis rate was determined using Annexin V and 7AAD according to the manufacturer's instructions (Annexin V, Alexa Fluor 488 conjugate, Invitrogen) in a BD Accuri™ C6 flow cytometer (BD Biosciences). The data were analyzed using the Flowjo v10.4 software (TreeStar, Inc.) and a total of 10,000 events were collected. Actinomycin D (Sigma, 80 μg/mL) was used as positive control.

Flow cytometry analysis of inflammatory cytokines

Before inflammatory cytokines levels evaluation, around 60,000 cells per/well were cultured to a confluence of 80 % on 24-well plates (Corning-Costar) and infected as described above. The inflammatory cytokines concentrations were determined using the commercial kit Human Inflammatory Cytokines (IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70), Becton Dickinson cytometric Bead Array (CBA) (BD Biosciences Cat # 551811) in a BD Accuri™ C6 flow cytometer (BD Biosciences) according to the manufacturer's instructions. CBA analysis was carried out using FCAP software (Becton-Dickinson).

Membrane abundance of TLR4

For TLR4 receptor membrane abundance (MA), 30,000 cells per/well were cultured at 80% confluence on 24 well plates (Corning-Costar) and infected as described above. Then the cells were detached with trypsin (5 mg/mL, Sigma), and the cell pellet was recovered by centrifugation (2500 rpm, 10 min, 4 °C), and washed two times with 100 μL of cold-PBS (pH 7.4). Cells were blocked during 30 min at 4 °C with horse serum (5 % in PBS, Pierce) and recovered by centrifugation. Furthermore, the cells were incubated with the primary antibody anti-TLR4 (sc-293072: Santa Cruz Biotechnology) diluted 1:100 in Phosphate Buffered Saline (PBS) containing 0.1% BSA (Bovine Serum Albumin) for 2 h at 4 °C with

shaking. Subsequently, the cells were incubated with an anti-rabbit IgG-Fab 2-PE conjugate as secondary antibody (1:50, Molecular Probes) for 1 h at 4 °C. The samples were analyzed in a BD Accuri™ C6 flow cytometer with the BD Accuri C6 software, and 10,000 events were collected and analyzed. HeLa cells stimulated with LPS (1 µg/mL, Sigma) for 24 h were used as positive control, while cells incubated only with the secondary as negative control.

Statistical analysis

Data were obtained from two independent experiments performed by triplicate, and were compared using the analysis of variance (ANOVA) in GraphPad Prism software (version 7, USA). Results are reported as means ± standard error (SE), and a p value <0.05 was considerate as positive.

RESULTS

Growth of *Gardnerella vaginalis*

In order to establish the infection model, first *G. vaginalis* strain was growth in thioglycollate and BHI. We observed similar growth behavior in both broths, the logarithmic phase starts from time 0 to 2 h while the stationary phase from 3 to 12 h. However, in thioglycolate broth an OD greater than 0.2 was not observed (**Fig. 1 panel A**). Further, we performed trypan blue exclusion assay to confirm the effect of bacterium infection over the cellular viability, at 2, 4 and 6 h post-infection with MOI 1 only 25% of cells died. While in MOI 20, after 2 h post-infection 30% of cells were death, 50% at 4 h and around the 90% at 6 h (**Fig. 1 panels B and C**). Finally, we decided to perform future experiments using only 2 and 4 h post-infection at MOI 1 and 20.

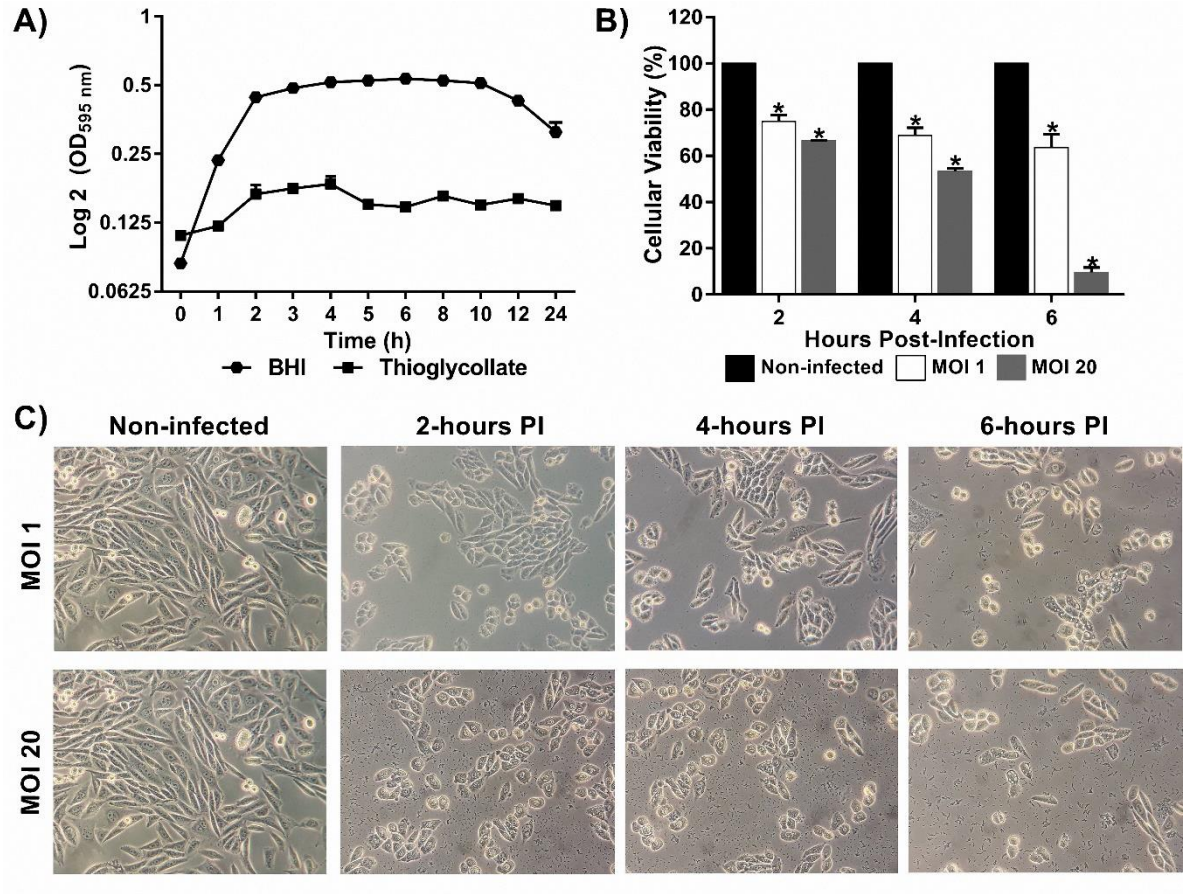

Figure 1. The infection with *Gardnerella vaginalis* ATCC 14018 affects HeLa cells viability. A) Growth curve of *G. vaginalis.* Bacterium was growth in thioglycollate and BHI broth at 37°C and 180 rpm. Cellular concentration was determined by OD at 595 nm. B) Trypan blue exclusion assay in HeLa cells infected with MOI 1 and MOI 20. In of all the experiment each point represents the mean and standard errors of two independent experiments performed by triplicates. (*) indicates significant *p* value changes ($p < 0.05$) compared to control cells (untreated cells). C) Graphic representation of cell morphology after infection with *G. vaginalis* ATCC 14018 (2, 4 and 6 h) MOI 1 and 20 (15×10^6 CFU/mL). PI (Post-infection).

***Gardnerella vaginalis* causes apoptosis in HeLa cells.**

Cells viability test showed that after infection with *G. vaginalis* around the 50 % of HeLa cells were dead, for this reason we studied the possible death mechanism. In the apoptosis induction assay, we observed 2.6 times greater apoptosis induction at 4 h post-infection with *G. vaginalis* at MOI 20, while in the cells infected during 2 h MOI 1 and 20 and 4 h MOI 1 no apoptosis was detected (**Fig. 2 panel A and B**).

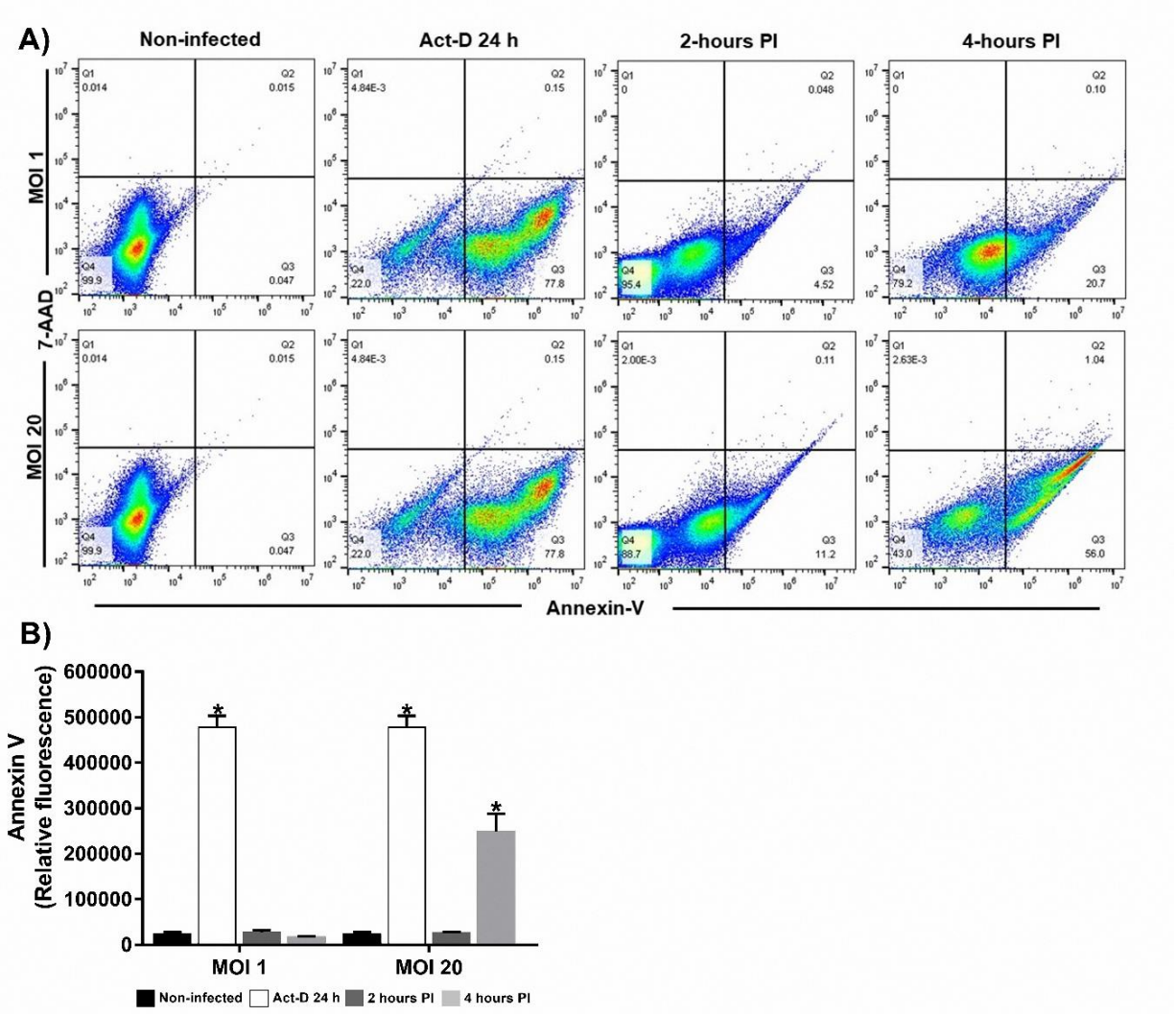

Figure 2. *Gardnerella vaginalis* induces apoptosis in HeLa cells. Hela cells were infected with *G. vaginalis* ATCC 14018 during 2 and 4 h. The apoptotic rate was determinated by flow cytometry using Annexin V/7AAD staining. The quadrants indicate viable cells (lower left quadrant), early apoptosis (lower right quadrant), late apoptosis (upper right quadrant) and necrotic cells (upper left quadrant). B) Relative fluorescence for each time of infection (relative units). Act-D (80 µl/ml) was used as a positive control. Each bar shows the mean ± error_standard of two independent experiments performed in triplicates. (*) indicates significant *p* value changes ($p < 0.05$) compared to control cells (untreated cells). PI (Post-infection).

***Gardnerella vaginalis* induces inflammatory cytokines expression**

Considering the established infection model, we proceeded to analyzed the effect of *G. vaginalis* infection on the secretion of inflammatory cytokines such as; TNF-α, IL-1β, IL-6, IL-12p70, IL-8 and IL-10 in HeLa cells. We observed a significant expression of IL-12p70 and IL-1β with MOI 1 and 20 at 2 and 4 h, on other hand, a significant production of IL-8 and IL-10 was observed with MOI 1 (IL-8 $p = 0.031$/IL-10 $p = 0.02$) only after 4 h post-infection, however, at MOI 20 after 2 h of incubation, a significant increase was detected which remained elevated until 4 h (IL-8 $p = 0.005/0.002$-IL-10 $p = 0.03/0.01$). Whereas, the secretion of IL-6 ($p = 0.02$) and TNF-α ($p = 0.03$) increased significantly only after 4 h of incubation with MOI 20 (**Fig. 3**).

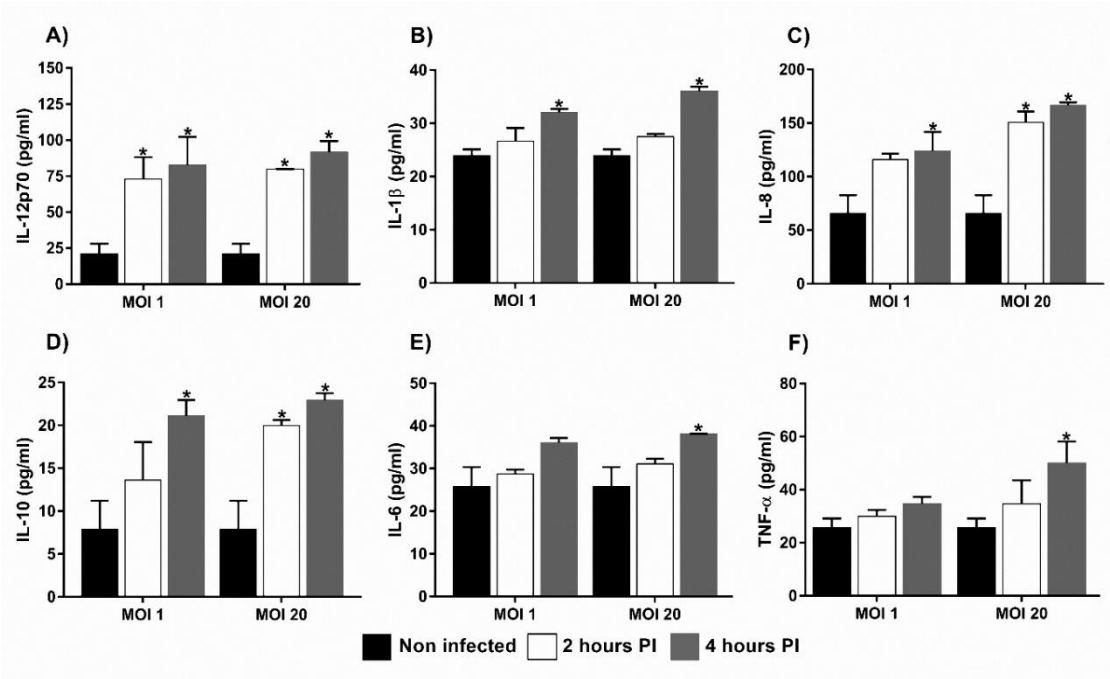

Figure 3. *Gardnerella vaginalis* induces cytokine secretion in HeLa cells. Cells were infected with *G. vaginalis* ATCC 14018 (2 and 4 h) with a multiplicity of infection (MOI) 1 and 20 (15 x 10^6 CFU/mL) and 300 events were collected and quantified. Analyzed cytokine were; IL12p70, TNF, IL-10, IL-6, IL-1β and IL-8 by flow cytometry. Each bar shows the mean ± errors standard from two independent experiments each performed in triplicates. (*) indicates significant *p* value changes ($p<0.05$) compared to control cells (untreated cells). PI (Post-infection).

The TLR4 membrane abundance is induced by *Gardnerella vaginalis*

The production of anti-inflammatory cytokine is a response to the activation of pattern recognition receptors (PRR) such as Toll-like receptor (TLRs), for this reason we analyzed the membranal abundance of TLR4. Results showed that after the infection with MOI 20 during 2 and 4 h membranal abundance of TLR4 increases around 2-folds, while in MOI 1 the increase was 1.5-folds only at 4 h post-infection (**Fig. 4**).

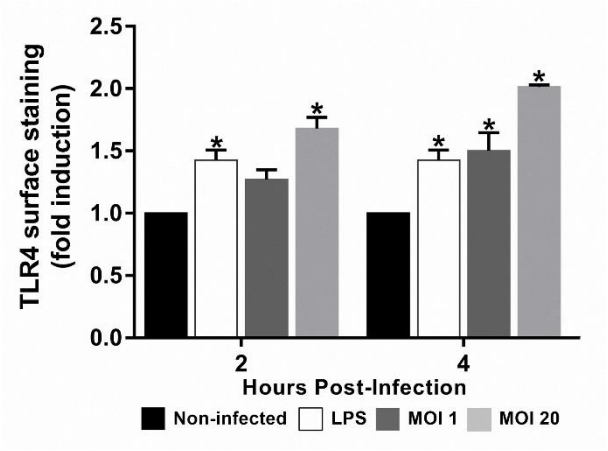

Figure 4. The TLR4 membrane abundance (MA) is regulated by the infection with *G. vaginalis* ATCC 14018. HeLa cells were challenged with *G. vaginalis* for 2 and 4 h. The receptor abundance was evaluated by flow cytometry. The fluorescence intensity was estimated from 10,000 events. LPS (1μg/ml) was used as a positive control. Each bar shows the mean ± error standard of two independent experiments performed in triplicates. (*) indicates significant *p* value changes ($p<0.05$) compared to control cells (untreated cells).

DISCUSSION

Bacterial vaginosis is a vaginal condition in women of reproductive aged, with a prevalence of 48.6% (21), being *Gardnerella vaginalis* the main etiologic agent (22). At immunological level BV is characterized by the lack of inflammatory cells, however, several studies performed in vaginal and cervicovaginal fluids of women with BV have reported high levels of inflammatory cytokines, nevertheless the participation of *G. vaginalis* during the modulation of the immune response has not been determinated (23).

After the infection with the bacteria, we observed that the cell viability decreased around 50%, probably due to the high pathogenic potential of the strain. It has been reported that *G. vaginalis* produces a cytolysin called vaginolysin (VLY) (24), ELISA assays have shown that the bacterium can produce up to 600 ng/mL of the toxin (25**),** which lyses epithelial cells through binding with the human CD59 (hCD59) receptor and cholesterol molecules present in cell membranes (10), in addition to others hydrolytic enzymes that promote the degradation by proteases and the female genital tract tissue destruction (26).

We determinated the apoptotic index in the infected cells, and observed that around the 42% of cells died by early apoptosis, these could be a consequence of VLY production which has been previously reported that induce IL-8 secretion and MAPK pathway activation triggering the apoptotic pathway and stress stimuli (27), in order to eliminate the microorganism and find a balance in the vaginal microenvironment (28**).**

Additionally, levels of inflammatory cytokines were quantified in the infected cells, and we observed a significant increased concentration of them (IL-12p70, IL-8, IL-10, TNF-α, IL-6 and IL-1β) at 4 h post-infection with MOI 20. These changes could be due to genetic

background (affecting the production of chemokines such as IL-8). Previously, *in vitro* models have demonstrated that *G. vaginalis* induces the production of pro-inflammatory cytokines (such as IL-1β, IL-8, IL-6, TNF-α) and anti-inflammatory cytokines (such as IL-5 and IL-10) (29,30). Particularly, the increase of IL-1β could be due to the elimination of the microorganism's virulence factors by the innate immune system (31), since this cytokine is crucial against the pathogens, nevertheless high levels can also enhance the acquisition of others infections (32). Cytokines production could be result of the hydrolytic enzymes productions such as sialidases and prolidases, that hydrolyze the sialic acid of the glycoproteins (including IgA immunoglobulin) and can potentially affect the extracellular matrix components, such as mucin. This mechanism plays an important role in the modulation of immune factors, in addition to deterioration and obstruction of the adaptive capacity and innate immune response in women with BV (33).

The production of short chain fatty acids (SCFAs) by the vaginal microbiota modulates the antimicrobial and immune activity, however this could be countered by the production of succinate by *G. vaginalis* which contributes to inhibit neutrophil chemotaxis (34, 35). In addition, *G. vaginalis* produces immunomodulatory substances such as prolidase and sialidases, that induce the inhibition inflammatory cell response (36). These findings suggest that the transition from normal vaginal microbiota to BV-associated microbiota, could be due to the production of certain pathogen associated molecular patterns (PAMPs) such as: Lipopolysaccharide (LPS), heat shock proteins (HSP), etc., and the immune response mechanism activated by pattern recognition receptors (PRR) between them Toll-like receptors (TLRs) (37).

A 42% of membrane abundance of TLR4 was found in the infected cells, which could be a consequence of the virulence factors that produce *G. vaginalis*, among them; sialidases, prolidases and VLY that are enzymes capable to increases adhesion, invasion and destruction of the cell infected, due to the osmotic imbalance, favoring access to the nutrients and promoting the remodeling of the extracellular matrix (10, 13, 38), releasing molecules as heat shock proteins, mainly HSP70, which can activate TLR4 to regulated signaling pathways such as p38 kinase, extracellular signal-related kinase (ERK), and nuclear factor kappa B (NF-κB), to secrete inflammatory cytokines, that will modulate the infection (39-41). It has been shown that high levels of this protein are found in cervicovaginal fluids of women with BV (42), so could be considered as a ligand for this receptor, however, additional studies need to be performed in order to demonstrate the specific ligand of TLR4 during the infection with *G. vaginalis*.

All of these findings, suggests that the microenvironment created by *G. vaginalis* characterized by the elevated vaginal pH, inflammatory cytokines production by TLR4 signaling, apoptosis induction and epithelial cell lysis, can affect to the vaginal and cervical epithelium, which is associated with squamous metaplasia arrest, epithelium damage, cervical mucus degradation, which could lead to a vaginal disorder and promote more susceptibility to acquire sexual transmission infections such as HIV, HPV, between other (30, 43**)**. Although, it is necessary to carry out further studies, in order to extrapolate this *in vitro* model to women with BV. We provide evidence about the role of *G. vaginalis* during the infection; its capacity to active inflammatory proteins production and apoptosis induction. Interest studies to carry on, will be quantify the expression of this cytokines in cytological

samples of women with and without BV, cell infection with other strains of *G. vaginalis*, and the potential role of this bacterium in the development of chronic inflammation, which will lead to acquiring coinfections that could be a risk factor to exacerbate endometritis, pelvic inflammatory disease and preterm birth and some type of carcinogenesis (44).

Taken together, our findings show that *G. vaginalis* could play an essential role in modulating the innate immune response during BV development. After infection, the bacterium stimulates the secretion of inflammatory cytokines such as IL-6, IL-8, IL-10, IL-1β, IL-12p70 and TNF-α, increase the membrane abundance of Toll-like receptors such as TLR4, and induce cellular death. Despite the fact that *G. vaginalis* colonize the vagina tract with other anaerobes, results shown the high pathogenic potential of the bacteria that along with others microorganism's virulence factors could lead to several complications in the female genital tract.

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DISCLOUSURE

The authors have no conflicts of interest to disclose.

REFERENCES

1. Schwebke J.R., Muzny C.A., Josey W.E. (2014). Role of *Gardnerella vaginalis* in the Pathogenesis of bacterial vaginosis: a conceptual model. *J infect Dis* **210**: 338-43.
2. Koumans E.H., Kendrick J., Sutton M., Bruce C., McQuillan G., Markowitz L. (2007). The Prevalence of Bacterial Vaginosis in the United States, 2001–2004; Associations with symptoms, sexual behaviors, and reproductive health. *Sex Transm Dis* **34**: 864–69.
3. Bautista C.T., Wurapa E., Sateren W.B., Morris S., Hollingsworth B., Sanchez J.L. (2016). Bacterial vaginosis: a synthesis of the literature on etiology, prevalence, risk factors, and relationship with chlamydia and gonorrhea infections. *Mil Med Res* **3**: 1-10.
4. Thulkar J., Kriplani A., Agarwal N., Vishnubhatla S. (2010). Aetiology & risk factors of recurrent vaginitis & its association with various contraceptive methods. *Indian J Med Res* **131**: 83–87.
5. Gardner H.L., Dukes, C.D. (1955). Haemophilus vaginalis vaginitis. A newly defined specific infection previously classified "nonspecific" vaginitis. *Am J Obstet Gynecol* **69**: 962- 76.
6. Baruah, F.K., Sharma, A., Das, C., Hazarika, N., Hussain, J.H. (2014). Role of *Gardnerella vaginalis* as an etiological agent of bacterial vaginosis. *Iran J Microbiol* **6**: 409-14.
7. Piot P., Dyck E.V., Peeters M., Hale J., Totten P.A., Holmes K.K. (1984). Biotypes of *Gardnerella vaginalis*. *J Clin Microbiol* **20**: 677-79.

8. Ingianni A., Petruzzelli S., Morandotti G., Pompei R. (1997). Genotypic differentiation of *Gardnerella vaginalis* by amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Immunol Med Microbiol*. **18**: 61-66.

9. Patterson J.L., Girerd P.H., Karjane N.W., Jefferson K.K. (2007). Effect of biofilm phenotype on resistance of *Gardnerella vaginalis* to hydrogen peroxide and lactic acid. *Am J Obstet Gynecol*. **197**: 170-77.

10. Randis T.M., Zaklama J., LaRocca T.J., Los F.C., Lewis E.M., Desai P., Rampersaud R., Amaral F.E., Ratner A.J. (2013). Vaginolysin drives epithelial ultrastructural responses to *Gardnerella vaginalis*. *Infect Immun*. **81**: 4544-50.

11. Sturm A.W. (1989). Chemotaxis inhibition by *Gardnerella vaginalis* and succinate producing vaginal anaerobes: composition of vaginal discharge associated with *G. vaginalis. Genitoriun Med* **65**: 109-12.

12. Africa C., Nel J., Stemmet M., (2014). Anaerobes and Bacterial Vaginosis in Pregnancy: Virulence Factors Contributing to Vaginal Colonization. *Int J Environ Res Public Health* **11**: 6979–7000.

13. Cauci S., Thorsen P., Schendel D.E., Bremmelgaard A., Quadrifoglio F., Guaschino S. (2003). Determination of immunoglobulin A against *Gardnerella vaginalis* hemolysin, sialidase, and prolidase activities in vaginal fluid: implications for adverse pregnancy outcomes. *J Clin Microbiol* **41**: 435-38.

14. Marconi C., Doncers G.G.G., Bellen G., Brown D.R., Parada C.M.G.L, Silva M.G. (2013). Sialidase activity in aerobic vaginitis is equal to levels during bacterial vaginosis. Eur *J Obstet Gynecol Reprod Biol* **167**: 205-09.

15. Ferreira C.S.T, Marconi C., Parada C.M.L.G., Duarte M.T.C., Gonçalves A.P.O, Rudge M.V.C., Da Silva M.C. (2015). Bacterial vaginosis in pregnant adolescents: proinflammatory cytokine and bacterial sialidase profile. Cross-sectional study. *Sao Paulo Med J* **133**: 465-70.

16. Masson L., Arnold K.B., Little F., Mlisana K., Lewis D.A., Mkhize N., Gamieldien H., Ngcapu S., Johnson L., Lauffenburger D.A., Abdool K.S.S., Passmore J.A.S. (2016). Inflammatory cytokine biomarkers to identify women with asymptomatic sexually transmitted infections and bacterial vaginosis who are at high risk of HIV infection. *Sex Transm Infect* **92**: 186-93.

17. Ryckman K., Williams S.M., Krohn M.A., Simhan H.N. (2008). Racial differences in cervical cytokine concentrations between pregnant women with and without bacterial vaginosis. *J Reprod Immunol* **78**: 166-71.

18. Hwang L.Y., Scott M.E., Ma Y., Moscicki A.B. (2011). Higher levels of cervicovaginal inflammatory and regulatory cytokines and chemokines in healthy young women with immature cervical epithelium. *J Reprod Immunol* **88**: 66-71.

19. Vick E.J., Park H.S., Huff K.A., Brooks K.M., Farone A.L., Farone M.B. (2014). *Gardnerella vaginalis* triggers NLRP3 inflammasome recruitment in THP-1 monocytes. *J Reprod Immunol* **106**: 67-75.

20. Goepfert A.R., Varner M., Ward K., Macpherson C., Klebanoff M., Goldenberg R.L., Mercer B., Meis P., Iams J., Moawad A., Carey J.C., Leveno K., Wapner R., Caritis S.N., Miodovnik M., Sorokin Y., O'Sullivan M.J., Van Dorsten J.P., Langer O. NICHD Maternal-Fetal Medicine Units Network. (2005). Differences in inflammatory cytokine

and Toll-like receptor genes and Bacterial vaginosis in pregnancy. *Am J Obstet Gynecol* **193**: 1478-85.

21. Bitew A., Abebaw Y., Bekele D., Mihret A. (2017). Prevalence of bacterial vaginosis and associated risk factors among women complaining of genital tract infection. *Int J Microbiol* **2017**: 1-8.
22. Gilbert N.M., Lewis W.G., Lewis A.L. (2013). Clinical features of bacterial vaginosis in a murine model of vaginal infection with *Gardnerella vaginalis. PLoS One* **8**: 1-13.
23. Boomsma C., Kavelaars A., Bozkurt N., Eijkemans M., Fauser B., Heijnen C., Macklon N.S. (2010). Is bacterial vaginosis associated with a pro-inflammatory cytokine profile in endometrial secretions of women undergoing IVF?. *Reprod Biomed Online* **21**: 133-41. doi: 10.1016/j.rbmo.2010.03.022.
24. Nowak R.G., Randis T.M., Desai P., He X., Robinson C.K., Rath J.M., Glover E.D., Ratner A.J., Ravel J., Brotman R.M. (2018). Higher levels of a cytotoxic protein, vaginolysin, in lactobacillus-deficient community state types at the vaginal mucosa. *Sex Transm Dis* **45**: 14-17.
25. Randis T.M., Kulkarni R., Aguilar J.L. Ratner A.J., (2009). Antibody-Based detection and inhibition of vaginolysin the *Gardnerella vaginalis* cytolysin. *Plos one* **4**: 1-5.
26. Cauci S., Guaschino S., Driussi S., De Santo D., Lanzafame P., Quadrifoglio F. (2002). Correlation of local interleukin-8 with immunoglobulin A against *Gardnerella vaginalis* hemolysin and with prolidase and sialidase levels in women with bacterial vaginosis. *J Infect Dis* **185**: 1614-20.

27. Gelber S.E., Aguilar J.L., Lewis K.L.T., Ratner A.J. (2008). Functional and phylogenetic Characterization of vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. *J Bacteriol* **190**: 3896-3903.

28. Campisi L., Cummings R.J., Blander M. (2014). Death-defining immune response after apoptosis. *Am J Transplant* **14**: 1488-98.

29. Bertran T., Brachet P., Vareille-Delarbre M., Falenta J., Dosgilbert A., Vasson M.P., Forestier C., Tridon A., Evrard B. (2016). Slight Pro-Inflammatory Immunomodulation Properties of Dendritic Cells by *Gardnerella vaginalis*: The "Invisible Man" of Bacterial Vaginosis. *J Immunol Res* **2016**: 1-13.

30. Aldunate M., Srbinovski D., Hearps A., Latham C., Ramsland P., Gugasyn R. (2015). Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis. *Front Physiol* **6**: 1-23.

31. Sturm-Ramirez K., Gaye-Diallo A., Elsen G., Mboup S., Kanki P. (2000). High levels of tumor necrosis factor-α and interleukin-1β in bacterial vaginosis may increase susceptibility to human immunodeficiency virus. *J Infect Dis* **182**: 467-73.

32. Forsum U., Holst E., Laesson P., Vasquez A., KJakobsson T., Mattsby-Baltzer I. (2005). Bacterial vaginosis-a microbiological and immunological enigma. *APMIS* **113**: 81-90.

33. Tafner Ferrerira, C., Marconi C., García Parada C., Cassamassimo Duarte M., Oliveira Goncalvez, A., Cunha Rudge, M. (2014). Bacterial vaginosis in pregnant adolescents: proinflammatory cytokine and bacterial sialidase profile. Cross-sectional study. *Sao Paulo Med J* **133**: 465-70.

34. Wiggins R., Hicks S.J., Soothill P.W., Millar M.R., Correld A.P. (2001). Mucinases and sialidases: their role in the pathogenesis of sexually transmitted infections in the female genital tract. *Sex Transm Infect* **77**: 402-08.

35. Sturm A.W. (1989). Chemotaxis inhibition by *Gardnerella vaginalis* and succinate producing vaginal anaerobes: composition of vaginal discharge associated with *G vaginalis. Genitourin Med* **65**: 109-12.

36. Marconi C., Santos-Greatti M.M.V., Parada C., Pontes A., Pontes A.G. Giraldo P., Donders G.G.G., Da Silva M.G. (2014). Cervicovaginal levels of proinflammatory cytokines are increased during chlamydial infection in bacterial vaginosis but not in Lactobacilli-Dominated Flora. *J Low Genit Tract Dis* **18**: 261-65.

37. Zhu J., Mohan C. (2010). Toll-like receptor signaling. Pathways-Therapeutic opportunities. *Mediators Inflamm* **2010**: 1-7.

38. Castro J., Âlves P., Sousa C., Cereija T., França A., Jeffeerson, K.K. (2015). Using an *in-vitro* biofilm model to assess the virulence potential of bacterial vaginosis or non-bacterial vaginosis *Gardnerella vaginalis* isolates. *Scientific Reports* **2015**: 1-10.

39. Kawai T., Shizuo A. (2010). The role of pattern-recognition receptors in innate immunity; update on Toll-like receptors. *Nat Immunol* **11**: 373-84. doi:10.1038/ni.1863.

40. Akira S., Uematsu S., Takeuchi O. (2006). Pathogen recognition and innate immunity. *Cell* **124**: 783-801.

41. Zhou J., An H., Xu H., Liu S., Cao X. (2005). Heat shock up-regulates expression of Toll-like receptor-2 and Toll-like receptor-4 in human monocytes via p38 kinase signal pathway. *Immunology* **114**: 522-30.

42. Giraldo P., Neuer A., Korneeva I.L., Ribeiro-Filho A., Simões J.A., Witkin S.S. (1999). Vaginal heat shock protein expression in symptom-free women with a history of recurrent vulvovaginitis *Am J Obstet Gynecol* **180**: 524-29.

43. Gillet E., Meys J.F.A., Verstraelen H., Verhelst R., De sutter P., Temmerman M. (2012). Association between Bacterial Vaginosis and Cervical Intraepithelial Neoplasia: Systematic Review and Meta-Analysis. *Plos One* **7**: 1-10.

44. Lu H., Jiang P.C., Zhang X.D., Hou W.J., Wei Z.H., Lu J.Q., Zhang H., Xu G.X., Chen Y.P., Ren Y., Wang L., Zhang R., Han Y. (2015). Characteristics of bacterial vaginosis infection in cervical lesions with high risk human papillomavirus infection. *Int J Clin Exp Med* **8**: 21080-88.

ABREVIATIONS

BV	Bacterial vaginosis
CFU	Colony Forming Units
ERK	Extracellular signal-related kinase (ERK)
HSP	Heat Shock Protein
LPS	Lipopolysaccharide
MOI	Multiplicity of Infection
NF-κB	Nuclear Factor kappa B
OD	Optical Density
SCFAs	Short chain fatty acids
TLR	Toll-like receptor

ANEXO

Expresión de IL-6, IL-10, COX-2 y TLR4 en citologías con y sin lesión escamosa intraepitelial de bajo grado de cérvix uterino.

La vaginosis bacteriana (VB) es una entidad clínica caracterizada por el reemplazo de la microbiota vaginal normal, como consecuencia de la colonización de microorganismos anaerobios. Estudios realizados en fluidos vaginales y cervicovaginales de mujeres con VB, han mostrado alteraciones en la expresión de proteínas inflamatorias, aunque la entidad clínica se caracteriza por la ausencia de células de la respuesta inmune, sin embargo, no se ha determinado si es debido al efecto de la infección por *G. vaginalis* al ser el principal agente causal, o al conjunto de los microorganismos anaerobios asociados (Marconi, *et al*., 2014). Cabe señalar, que esta alteración en el tracto genital femenino, ha sido asociado con un mayor riesgo de desarrollar coinfecciones, como infecciones de transmisión sexual (Gillet *et al*., 2011; Audirac-Chalifour *et al*., 2016).

MATERIALES Y MÉTODOS

Población de estudio

En este estudio, participaron 116 mujeres residentes del estado de Guerrero, México, que acudieron al servicio de detección oportuna de Cáncer Cervicouterino (CaCu) e infección por VPH. El protocolo fue aprobado por el Comité de Ética de la Universidad Autónoma de Guerrero, y todas las participantes firmaron un consentimiento informado con base a lo estipulado por la declaración de Helsinki, 2013. Adicionalmente, se obtuvieron datos clínicos, demográficos y sociales por medio de una encuesta.

Toma de muestra

Las muestras fueron tomadas del endo-exocervix con una espátula de Ayre (ectocervix) y con un citobrush (endocérvix), asegurando la obtención de material citológico de la zona de transformación escamocolumnar. Los extendidos citológicos fueron utilizados para el análisis citomorfológico, usando la tinción convencional de Papanicolaou y la citología en base liquida-PREP^{TM} (LPT). El diagnóstico citológico fue realizado por un citotecnólogo certificado en base a la clasificación de Bethesda (Nayar and Wilbur, 2015). Para el diagnóstico de VB se consideraron los criterios de Amsel, que consiste en la evaluación del pH vaginal >4.5, flujo

vaginal, prueba de aminas positiva y presencia de células clave (Amsel *et al.,* 1983). Mientras que, para la identificación y genotipificación el VPH-AR se utilizó el kit INNO-Lipa genotyping Extra (Innogenetics). Tomando en consideración el diagnóstico de VB, el citológico y la presencia del VPH-AR, las muestras se clasificaron en 6 grupos; 1) Sin LEI/Sin VB/Sin VPH (n=26), 2) Sin LEI/Sin VB/Con VPH-AR (n=15), 3) Sin LEI/Con VB/Sin VPH (n=15), 4) Sin LEI/Con VB/Con VPH-AR (n=3), 5) LEIBG-VPH-AR/Sin VB (n=30) y 6) LEIBG-VPH-AR/Con VB (n=27).

Expresión de IL-6, IL-10, TLR4 y COX-2 por inmunocitoquímica

La expresión de las proteínas en muestras citológicas, se determinó por el método inmunocitoquímico biotina/estreptavidina-peroxidasa, utilizando el sistema de detección celular Cytoscan HRP/DAB (Cell Marque Corporation, Hot Springs, AR, USA). Se utilizaron los anticuerpos: anti-IL-6 (M-19: sc-1265; 1:100; Santa Cruz Biotechnology, Inc), anti-IL-10 (E-10:sc-8438;1:100; Santa Cruz Biotechnology, Inc), anti-TLR4 (25:sc-293072;1:100; Santa Cruz Biotechnology, Inc) y anti-COX-2 (H-62:sc-795;1:100; Santa Cruz Biotechnology, Inc). Las muestras fueron sometidas a recuperación antigénica (Immuno DNA Retriever con Declere, Bio SB Inc., Santa Barbara, CA, USA) por 6 min a 120°C. Se incubó con el anticuerpo primario durante 2 h, con biotina 40 min, y estreptavidina 30 min. Finalmente, para evidenciar la reacción antígeno-anticuerpo, se utilizó como cromógeno diaminobenzidina (DAB) durante 1 min y hematoxilina de Mayer como colorante de contraste. Una muestra con cáncer cervical, que sobreexpresa estas proteínas, fue utilizada como control positivo, mientras que, como control negativo se utilizó la misma muestra, omitiendo el anticuerpo primario. Para la interpretación de los resultados obtenidos se evaluó la intensidad de la inmunotinción de acuerdo a las siguientes categorías; negativo, leve, moderado e intenso (Allred *et al*., 1998).

RESULTADOS

Expresión de proteínas inflamatorias y su relación con la infección por *Gardnerella vaginalis*

Los resultados de la inmunocitoquímica evidenciaron que en las muestras pertenecientes al grupo sin infección viral pero que están asociadas a VB (Sin LEI/ Sin VPH/Con VB) evidenciaron una expresión leve de COX-2 e IL-10. Mientras que, en los grupos con lesión cervical, asociados (LEIBG-VPH-AR con VB) y no asociados a VB (LEIBG-VPH-AR sin VB) se observó una expresión intensa de COX-2 e IL-10 en el 100% de las células, sin embargo, en el caso de IL-6 y TLR4, una expresión leve fue observada en el 100% de las células en los mismos grupos **(Tabla 1)**.

Tabla 1. Expresión de COX-2, IL-6, IL-10 y TLR4 en citologías cervicales

	Sin LEI/Sin VB/Sin VPH n (%)	Sin LEI/Sin VB/Con VPH n (%)	Sin LEI/Con VB/Sin VPH n (%)	Sin LEI/Con VB/Con VPH n (%)	LEIBG sin VB n (%)	LEIBG Con VB n (%)	TOTAL	Valor de *p*
Cox-2								
Negativo	26 (100)	0(0)	15 (100)	0(0)	0(0)	0(0)	41(35.3)	
Leve	0(0)	15 (100)	0(0)	0(0)	0(0)	0(0)	15(12.9)	**<0.0001**
Moderado	0(0)	0(0)	0(0)	3 (100)	0(0)	0(0)	3(2.6)	
Intenso	0(0)	0(0)	0(0)	0(0)	30 (100)	27 (100)	57 (49.1)	
Total	26 (100)	15 (100)	15 (100)	3 (100)	30 (100)	27 (100)	116 (100)	
IL-6								
Negativo	26(100)	15(100)	15 (100)	3 (100)	0(0)	0(0)	59(50.9)	
Leve	0(0)	0(0)	0(0)	0(0)	30(100)	27(100)	57(49.1)	**<0.0001**
Moderado	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	
Intenso	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	
Total	26(100)	15(100)	15(100)	3(100)	30(100)	27(100)	116(100)	
IL-10								
Negativo	26(100)	0(0)	0(0)	0(0)	0(0)	0(0)	26 (22.4)	
Leve	0(0)	15(100)	15(100)	0(0)	0(0)	0(0)	30(25.9)	**<0.0001**
Moderado	0(0)	0(0)	0(0)	3(100)	0(0)	0(0)	3(2.6)	
Intenso	0(0)	0(0)	0(0)	0(0)	30(100)	27(100)	57(49.1)	
Total	26(100)	15(100)	15(100)	3(100)	30(100)	27(100)	116(100)	
TLR4								
Negativo	26 (100)	15(100)	15(100)	3(100)	0(0)	0(0)	59(50.9)	
Leve	0(0)	0(0)	0(0)	0(0)	30(100)	27(100)	57(49.1)	**<0.0001**
Moderado	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	
Intenso	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	
Total	26(100)	15(100)	15(100)	3(100)	30(100)	27(100)	116(100)	

Los datos son reportados en n y (%). Valor de *p*: Xi^2

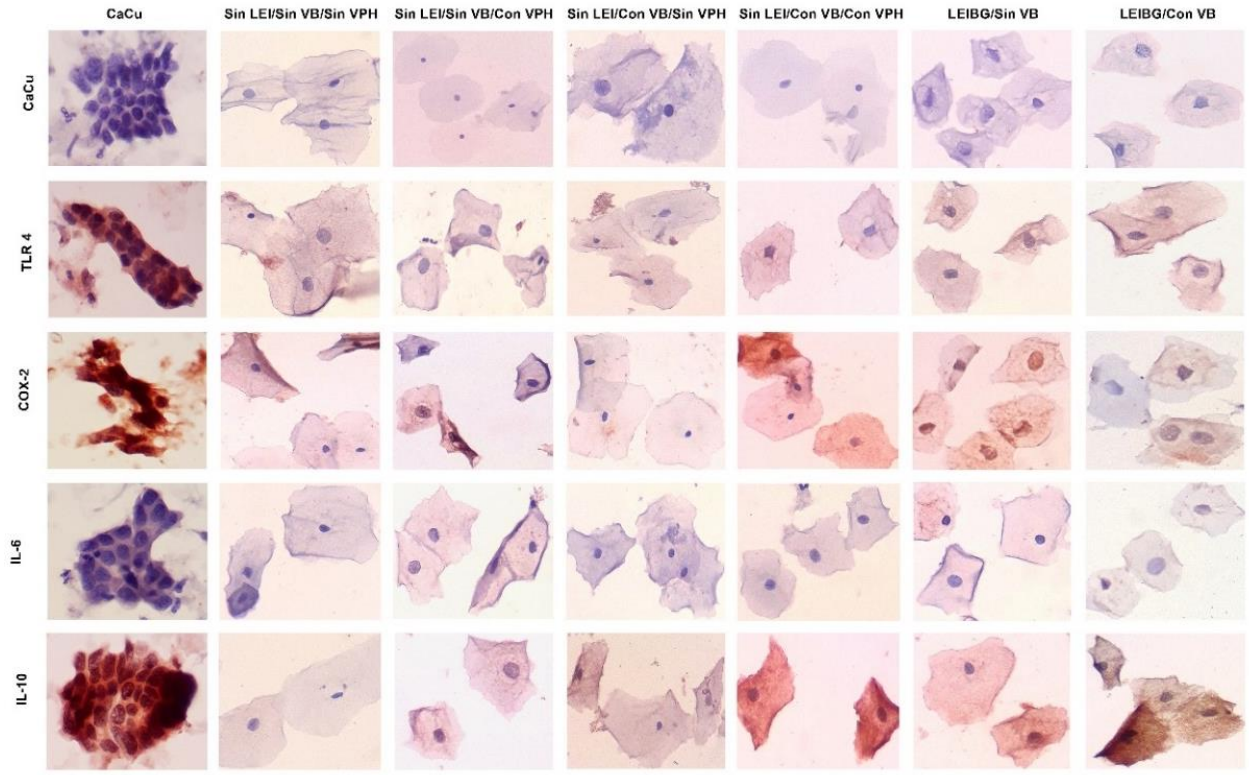

Figura 1. Determinación de la expresión de TLR4, Cox-2, IL-6 e IL-10 en muestras citológicas. 1) Control; Citología con Cancer Cervicouterino (CaCu), 2) Citología Sin LEI/Sin VB/Sin VPH, 3) Citología Sin LEI/Sin VB/Con VPH, 4) Citología Sin LEI/Con VB/Sin VPH, 5) Citología Sin LEI/Con VB/Con VPH, 6) Citología con LEIBG/ Sin VB, y 7) Citología con LEIBG/Con VB. Técnica: Inmunocitoquímica. Aumento 40X en microscopio óptico.

DISCUSIÓN

Gardnerella vaginalis es el principal agente etiológico aislado en mujeres con VB, debido a su alto potencial patogénico, causa microabrasiones y alteraciones en el balance inmunológico (Platz-Christensen *et al.*, 1993; Ryckman *et al.*, 2011). Sin embargo, no está determinado si existirá una diferencia en la expresión de proteínas pro y anti-inflamatorias en muestras citológicas sin lesión y con lesión escamosa intraepitelial de bajo grado con infección por VPH-AR que están asociadas y no asociadas a VB.

Se observó una sobreexpresión de IL-10 y COX-2 en el grupo de Sin LEI/Con VB/Con VPH-AR (100%) y LEIBG/VPH-AR con (100%) y sin VB (100%) **(Tabla 1).** Sin embargo, esto no puede ser atribuido a la presencia de *G. vaginalis*, sino a que el aumento en el pH vaginal durante el padecimiento de VB, favorece el arresto de metaplasia escamosa, incrementando la susceptibilidad de la zona de transformación para agentes oncogénicos, ya que, la infección al

estar ligada con la inhibición de la quimiotaxis de neutrófilos, que surge de los efectos de los ácidos succínicos y acéticos, favorece el desarrollo de coinfecciones como las infecciones de transmisión sexual, principalmente por VPH (Peres *et al.*, 2015), el cual a través de la interacción de sus oncoproteínas E6 y E7 puede inducir la activación del eje inflamatorio COX-prostaglandinas, elevando la expresión del oncogén inmediato COX-2 (Adefuye and Sales, 2012). En el caso de IL-10, al ser producida por las células del estroma, la hace tolerante al sistema inmune, y, por lo tanto, ser un factor para el desarrollo de lesiones premalignas (Giannini, 1998). Previamente se ha reportado una sobreexpresión de estas proteínas en biopsias de pacientes con lesiones premalignas y cáncer cervical, lo que se ha asociado a un estado de inmunosupresión local, así mismo, se ha propuesto que la sobreexpresión de COX-2 puede favorecer el desarrollo y la progresión de las lesiones intraepiteliales escamosas (Balan *et al*., 2011; Wang *et al*., 2013). Sin embargo, a pesar de que las infecciones bacterianas del tracto genital típicamente inducen una respuesta inflamatoria local, en nuestro estudio, no observamos expresión de las proteínas proinflamatorias (IL-6 y TLR4), cabe señalar, que puede ser resultado de mecanismos inmunes reguladores, como apoptosis de neutrófilos, lo que consiste eventualmente en la supresión de la producción de citocinas proinflamatorias, lo cual evita la resolución de la infección, y por consiguiente, favorece el desarrollo de lesiones premalignas (Weissenbacher *et al*., 2010; Scott *et al*., 2013).

CONCLUSIÓN

Se observó expresión IL-6, IL-10, TLR4 y COX-2, en los grupos sin y con LEIBG/VPH-AR con VB, por lo que, *G. vaginalis*, no podría considerarse el único agente etiológico capaz de modular la secreción de proteínas inflamatorias durante la VB.

REFERENCIAS

1. Gillet, E., Meys, J., Verstraelen, H., Bosire, C., De Sutter, P., Temmerman, M., *et al*. (2011). Bacterial vaginosis is associated with uterine cervical human papillomavirus infection: a meta-analysis. *BMC Infect Dis*. 11(10): 1-9.
2. Audirac-Chalifour, A., Torres-Poveda, K., Bahena-Román, M., Téllez-Sosa, J., Martínez-Barnetche, J., Cortina-Ceballos, B., *et al*. (2016). Cervical microbiome and cytokine profile at various stages of cervical cancer: A pilot study. *PLOS one*.11(4): 1-24.
3. Nayar, R., Wilbur, D. (2015). The Pap Test and Bethesda 2014. Acta cytologica.121-132.
4. Allred, D.C., Harvey, J.M., Berardo, M., Clark, G.M. (1998). Prognostic and predictive factors in breast cancer by immunohistocheical analysis. *Modern Pathology*.111(2). 155-168.
5. Platz-Christensen, J.J., Mattsby-Baltzer, I., Thomsen, P., Wiqvist, N. (1993). Endotoxin and interleukin-1α in the cervical mucus and vaginal fluid of pregnant women with bacterial vaginosis. *American J Obstet Gynecol*. 169(5): 1161-1166.
6. Ryckman, K.K., Williams, S.M., Krohn, M.A., Simhan, H.N. (2011). Interaction between interleukin-1 receptor 2 and Toll-like receptor 4, and cervical cytokines. *J Reprod Immunol.* 90(2): 220-226.
7. Peres, A.L., Camarotti, J.R.S.L., Cartaxo, M., Alencar, N., Stocco, R.C., Becak, W., *et al*. (2015). Molecular analysis and conventional cytology: association between HPV and bacterial vaginosis in the cervical abnormalities of brazilian population. *Genet Mol Res*. 14(3): 9497-95.
8. Adefuye, A., Sales, K. (2012). Regulation of inflammatory pathways in cancer and Infectious Disease of the cervix. *Scientifica (Cairo)*: 1-8.
9. Gianni, S.L., AL-Saleh, W., Piron, H., Jacobs, N., Doyen, J., Bonvier, J., *et al*. (1998). Cytokine expression in squamous intraepithelial lesions of the uterine cervix: implications for the generation of local immunosuppression. *Clin Exp Immunol*. 113(2): 183-189.

10. Balan, R., Amalinei, C., Giusca, S., Ditescu, D., Gheorghita, V., Crauciuc, E. (2011). Immunohistochemical evaluation of COX-2 expression in HPV-positive cervical squamous intraepithelial lesions. *Rom J Morphol Embryol*. 52(1): 39-43.
11. Wang, Y., Liu, H., Li, Y., Li, O. (2013). The paradox of IL-10 mediated modulation in cervical cancer (Review). *Biomed Rep*.1(3): 347- 351.
12. Weissenbacher, T., Walter, C., Mylonas, I., Scholz, C., Gingelmaier, A., Friese, K. (2010). Interleukin-6, interleukin-10 and interleukin-12 in vaginal fluid from women with bacterial vaginosis. *Arch Gynecol Obstet*. 281(1): 77-80.
13. Scott, M.E., Shvetsov, Y.B., Thompson, P.J., Hernández, B.Y., Zhu, X., Wilkens, L., *et al*. (2013). Cervical cytokines and clearance of incident human papillomavirus infection: Hawaii HPV cohort study. *Int J Cancer*. 133(5): 1187-1196