Evaluation of Topical Microbicides for their Acute Toxicities and Ability to Induce Proinflammatory Cytokines TNF-a, IL-1b, IL-6 and IL-8 in Peripheral Blood Mononuclear Cells and Macrophages

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Abstract

Purpose: Topical Microbicides have been used as vaginal microbicides in clinical trials as anti-HIV-1 products. The objectives of this project were to evaluate selected popular topical microbicide drug products for their acute toxicities to colorectal epithelial cells and primary immune cells such as Peripheral Blood Mononuclear Cells (PBMC) and Macrophages and to further determine if these microbicide products could induce genital tract inflammation via extra production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8.

Methodology: Toxicities of Nonoxynol-9, KY jelly, CAP, PRO2000 (4%), PRO2000 (0.5%), UC781(1%), UC781(0.1%) and VenaGel™ microbicides were determined by cellular viability of colorectal adenocarcinoma cells (CaCo$_2$), the model vaginal epithelial cell and the vaginal tract primary immune cells of peripheral blood mononuclear cells (PBMCs) and Macrophages by culturing them for 24-hours in the presence of serial dilutions of products or placebo. Products and placebo dilutions that gave culture viabilities of ≥60% compared to control cultures were considered as nontoxic

Results: The results indicated safety and low toxicity of these microbicides in terms of cytokine release. Furthermore, it showed that usage of these microbicides as anti-HIV formulation will not trigger cytokine release. However, IL-8 has shown relatively higher levels for all microbicides tested in toxic and nontoxic formulation. This indicated that microbicides induced the release of IL-8 and could trigger the recruitment of HIV-1 susceptible cells and increase HIV-1 replication.

Keywords: Nonoxynol-9; KY Jelly; CAP; Cytokines; TNF-a; IL-1b; IL-6 and IL-8

Introduction

Despite the success of HIV intervention methods, the HIV epidemic continues throughout the world and is accelerating in many areas. As at the end of 2020, there were 37.7 million (30.2 - 45.1 million) people living with the disease globally [1]. Furthermore, it has been estimated that the number of people living with HIV will continue to increase due to the ongoing accumulation of new infections [2]. However, though antiretroviral therapy (ART) is currently the most common treatment for HIV/AIDS, and can reduce viral load to undetectable levels, it does not cure the condition. Moreover, ART is expensive, requires a strict medication regime and is subject to social stigma. Therefore, even though, as many as 27.5 million of the people living with the

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infection has access to treatment, there are still about 10.2 million who were not on treatment [1]. Therefore, it has been suggested that a combination of prevention and treatment modalities may be necessary for the success of any global program to control the infection [3].

One of the promising prevention methods is using topical microbicides which are applied to mucosal surfaces to prevent HIV infections [2,5]. Such drug products containing microbicides for vaginal and rectal application can be self-administered pre-exposure prophylactic medications that can be applied to protect against sexually transmitted disease including HIV. Most microbicides candidates act by disrupting the cell membrane of the pathogen, blocking receptor-ligand interaction, or modifying the vaginal environment [6,7]. Therefore, for a microbicide drug product to meet the quality requirement, the agents must be safe following vaginal and rectal administration and cause minimal or no genital adverse effects after repeated use [8]. Given the expected long term exposure of the cervico-vaginal and rectal mucosa, the microbicide drug product should not display tissue toxicity at the drug concentrations and repeated applications intended to be used in humans [9]. Clinical experience of deleterious mucosal effect of nonoxynol-9 have shown that cytotoxic compounds that disrupt the mucosal tissues and normal flora can rather enhance HIV transmission [9,10]. It is, therefore, essential that such products be evaluated for safety in both urogenital and rectal tissues.

Cytotoxicity involves alteration of cell viability, multiplication and/or function. For anti-HIV microbicides, the toxicity should be evaluated on epithelial cells such as $CaCo_2$ that model cervical, vaginal and rectal cells. Colorectal adenocarcinoma cells ($CaCo_2$) are widely used as epithelial cell models for studies in drug discovery, particularly for mechanisms underlying toxicology, analysis of the interaction of drugs with epithelia membrane in absorption and metabolism [11]. Furthermore, given that immune cells are essential component in mucosal transmission of HIV, the impact of microbicides on these cells through any harmful effect of the drug products on epithelial cells, need to be determined [12]. Furthermore, it is important to investigation if these interactions of the drug products and immune cells can induce extra deleterious extra proinflammatory cytokines.

Currently, several microbicide drug products and candidates have been approved or at different stages of development. These include products that disrupt or inactivate the pathogen (e.g. nonoxymol-9, cellulose acetate phthalate [CAP] - disrupt the cell membrane of HIV or change the cell membrane structure to make it more porous and more liable to disruption); block pathways that allow fusion of pathogens to healthy cells (e.g., carrageenan), and affect the pathogen replication and life cycle (e.g., dendrimers, UC781-reverse transcriptase inhibitors) [4,8]. Alternatively, the formulation of a product may contain drugs that maintain or enhance vaginal defense mechanism (e.g., antimicrobial peptides such as D2A21 in Vena gel) or just an active excipient like Carbopol or polycarbophil, such as PRO 2000, and UC781. As such, the placebos for PRO 2000 and UC781 have been shown to have some anti-HIV-1 activity [12-14].

The candidate topical microbicides used in this project were products designed for use in humans and are as following: Thiocarboxianalide (UC781) is a thiocarboxanalide pentenloxy derivative of the carboxanilide class of compounds; Cellulose Acetate Phthalate (CAP) is a pharmaceutical excipient that has been used for over four decades for enteric film coating of tablets and capsules. CAP contains 13% polyanionic polymer with antimicrobial activity; Nonoxynol-9 (N-9) has been used for >30 years, in the United States and other countries, and it continues to be included in numerous spermicidal and lubricant products; Naphthalene sulphonate (PRO 2000) is a naphthalene sulfonic acid polymer; Carraguard is a water-solution mixture of sulfated polysaccharides (3%) extracted from red seaweed (Rhodophyceae), or Irish moss, and is found off the Atlantic coast; Vena Gel is a formulation containing a synthetic antimicrobial peptide (D2A21). The objectives of this study were to evaluate selected popular topical microbicide drug products on the market for their acute toxicities to colorectal epithelial cells and primary immune cells - Peripheral Blood Mononuclear Cells (PBMC) and Macrophages and to further determine if these microbicide products could induce genital tract inflammation via extra production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8 above the baseline vaginal content of these cytokines.

Materials and Methods

Materials

Cell culture

Colorectal adenocarcinoma, Caco-2 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The

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cells were grown in DMEM (C-DMEM) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 g/ml streptomycin, 100 U/ml penicillin, and 100 mM L-glutamine and maintained at 37°C, 7% CO_2 [4,15]

Human PBMCs were obtained from HIV-1 negative donors by leukophoresis. The cells were CD8-depleted (Dynal, Lake Success, NY) according to the manufacturer's instructions. The PBMCs were stimulated in C-RPMI media supplemented with 10% IL-2 and 1 µg/ml phytohemagglutinin-P at 37°C, 7%CO_2 for 3 days after which they were washed and suspended in the media.

Macrophages were obtained from human monocytes (Advanced Biotechnologies, Inc., Columbia, MD). The macrophages were maintained and cultured in DMEM supplemented with 20% heat-inactivated fetal calf serum, 10% human AB serum, 100 µg/ml streptomycin, 100 U/ml penicillin, and 100mM L-glutamine at 37°C, 7% CO_2.

Topical microbicides

KY® plus Nonoxynol-9 (KY-N9 - Ortho-McNeil Pharmaceuticals, Inc., Raritan, NJ) and K-Y® Jelly (McNeil-PPC, Inc., Skillman, NJ) are approved over the counter (OTC) products. UC781 (0.1%, 1% formulations and their Carbopol aqueous gel placebo were provided by Biosyn, Inc. (Huntingdon Valley). Micronized cellulose acetate phthalate (CAP) was provided by the Lindsley F. Kimball Research Institute (New York, NY) and Dow Pharmaceutical Sciences (Petaluma, CA), as Aquateric (containing about 66% micronized CAP; FMC Biopolymer Corp., Philadelphia, PA and 34% Poloxamer and distilled acetylated monoglycerides. CAP contains 13% polyanionic polymer with antimicrobial activity). Vena Gel™ and a hydroxymethyl cellulose placebo were provided by Demegen, Inc. (Pittsburgh, PA). PRO 2000 (0.5%, 4% and their Carbopol aqueous gel placebo formulations) were obtained from Indevus Pharmaceuticals, Inc (Lexington, MA).

Methods

Evaluation of toxicities

Toxic concentrations of the products and placebos were determined by culturing the Caco-2 cells for 24-hours in serial dilutions of products or placebos in 96-well plates at 5 x 10⁴ cells per well (Table 1). Cells incubated in only cell medium served as controls. Cell viability was determined by cellular ATP

concentrations using the CellTiter-Glo™ assay (Promega Corp., Madison, WI). After 24 hours incubation, the product and the media removed, and the wells were washed twice with fresh media. 500 µl of reconstituted CellTiter-Glo™ reagent were added into the wells of the plate. The plates were rocked to mix for 30 minutes before quantification. The quantification was by measuring the ratios of the Relative Light Units (RLU) between the test cultures and control from the ATP-dependent luminescence using DYMNEX MLX (Franklin, MA). Product and placebo dilutions that gave culture viabilities of ≥ 60% compared to control cultures were nontoxic and evaluated further.

Primary immune cells "PBMCs and macrophages" viability assay

Activated PBMCs and macrophages were separately incubated for 24 hours in serial dilutions of the products or placebo in medium, or medium alone (control) in 24-well plates at 1x10⁶ cells per well (Table 1). All test and control cultures were set-up in triplicate. 100 µl of culture media was removed for testing after the 24-hours incubation period. Cell viability was determined as described above.

Cytokines assays in PBMCs and macrophages

The products were diluted in the appropriate cell culture media. The dilutions depended on the strengths of individual drug products and results of the initial viability studies. The PBMCs and macrophages were incubated for 4 hours at a concentration of (1 x 10⁶ per well) in the medium containing (1.0ml media/well) toxic and nontoxic concentration of product or placebo or media alone (controls) for 4-hours (Table 1). Each product, placebo, and control were tested in triplicate. Culture supernatants were stored at minus 80°C until they were assayed for pro-inflammatory cytokines using Human Quantikine® HS cytokines immunoassay kit (R & D Systems, Minneapolis, MN).

Results

### Microbicide toxicities to epithelial CaCo₂ cell cultures

Overall, the products had varied toxicity levels to CaCo₂ cells (Table 1; Figure 1). Carraguard® (PC515) showed the least toxicity level to the cells. The toxic concentration was less than 1:10 (Table 1; Figure 1a). Nontoxic or cell viability concentrations of cellulose acetate phthalate (CAP) (Figure 1b), PRO 2000 (0.5%

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and 4%) (Figure 1c), and UC781 (0.1% and 1%) (Figure 1d) ranged from 1:25 to 1:50. This showed a mild cell toxicity of CaCo$_2$ cells to these products with CAP and PRO 2000 showing cell viability at concentrations of 1:25 and UC781 at 1:50. In contrast, nontoxic concentrations of Vena Gel™ (Figure 1e) and N9 (Figure 1f) were 1:1000. These showed 20- to 100-fold more toxicity to CaCo$_2$ cell than the other four products.

Cell Viability Studies – Nontoxic Dilution (Viability > 60%)			
Product	Caco2 Cells	PBMC	Macrophage
	Product Dilution/(Strength of Active Ingredients)		
VenaGel 1%	1:1000/(0.001%)	1:1000/(0.001%)	1:1000/(0.001%)
Placebo-Hydroxymethyl cellulose 3.25%	1:100/(0.0325%)	1:100/(0.0325%)	1:100/(0.0325%)
1% UC781	1:50/(0.02%)	1:20/(0.05%)	1:20/(0.05%)
0.1% UC781	1:50 (0.002%)	1:20/(0.005%)	1:20/(0.005%)
Placebo – Carbomer 1%	1:50/(0.02%)	1:20/(0.05%)	1:20/(0.05%)
KY Jelly	1:100/(0.1%)	1:100/(0.1%)	1:100/(0.1%)
N9 2%	1:1000/(0.002%)	1:1000/(0.002%)	1:1000/(0.002%)
4% PRO2000	1:25/(0.16%)	1:25/(0.16%)	1:20/(0.2%)
0.5% PRO2000	1:25/(0.02%)	1:10/(0.05%)	1:20/(0.025%)
Placebo – Carbomer 1%	1:10/(0.1%)	1:25/(0.04%)	1:20/(0.05%)
CAP (13% antimicrobial polyanionic polymer)	1:25/(0.52%)	1:100/(0.13%)	1:50/(0.26%)
Methyl Cellulose	1:5/(0.2%)	1:100/(0.1%)	1:5/(0.2%)
Carraguard™ (PC515)	1:5/(0.2%)	1:10/(0.1%)	1:5

Table 1: Nontoxic dilutions of the candidate microbicide and placebo formulations in epithelial cell lines and primary immune cells.
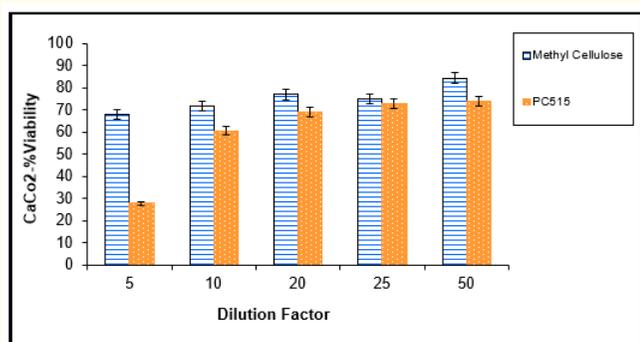
Figure 1a: Comparative CaCo$_2$ cell viabilities at the various dilutions of CarraguardTM (PC515) and its Methyl Cellulose placebo.
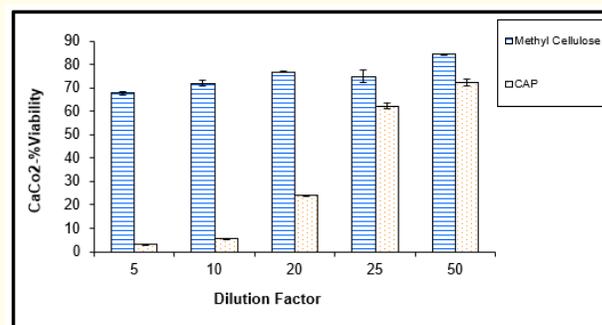
Figure 1b: Comparative CaCo$_2$ cell viabilities at the various dilutions of Cellulose Acetate Phthalate (CAP) and its Methyl Cellulose placebo.
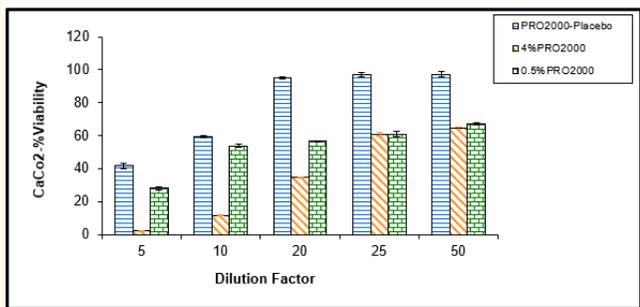
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Figure 1c: Comparative $CaCo_2$ cell viabilities at the various dilutions of 4% and 0.5% PRO 2000 and the placebo.

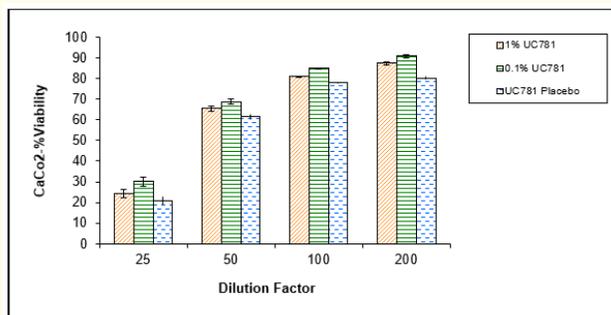**Figure 1d:** Comparative $CaCo_2$ cell viabilities at the various dilutions of 1% and 0.1% UC781 and the placebo.
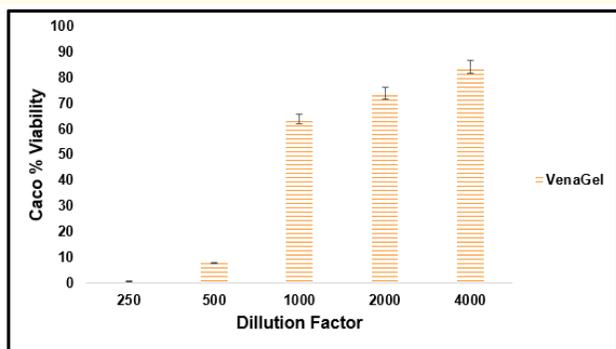
Figure 1e: Comparative $CaCo_2$ cell viabilities at the various dilutions of VenaGel.


Figure 1f: Comparative $CaCo_2$ cell viabilities at the various dilutions of KY Jelly and KY-N9.

The nontoxic dilutions of the five placebos ranged from 1:5 for methyl cellulose to 1:100 for the Vena Gel placebo and KY Jelly. The nontoxic dilution of PRO 2000 was 2.5 time greater than its placebo, and that of the UC781 formulations were equal to the placebo. This shows that most of the toxicities in these products were due to the excipients. In contrast, the nontoxic dilutions of N9 and Vena Gel™ were 10 times greater than those of their respective placebo formulations. This is an indication that most of the toxicities of these products were due to the active drug ingredients in the products rather than the base formulations. No additional toxicity occurred when nontoxic dilutions of the products and placebos were applied to epithelial cell cultures for 2-hours each day for 5-days.

Microbicide toxicities to the peripheral blood mononuclear cells (PBMCs)

The products toxicities to PBMCs followed a similar pattern to that of the $CaCo_2$ cells (Table 1; Figure 2). As in the $CaCo_2$ cells, the Carraguard® (PC515) showed the least toxicity level to the PBMCs. However, the nontoxic concentration was 1:10 which is two times more than to the $CaCo_2$ cells (Table 1; Figure 2a). Nontoxic or cell viability concentration of cellulose acetate phthalate (CAP) (Figure 2b) to the PBMCs was 1:100. This concentration is four times more than to the $CaCo_2$ cells (Table 1). Nontoxic or cell viability concentrations of and PRO 2000 (0.5% and 4%) (Figure 2c) and UC781 (0.1% and 1%) (Figure 2d) were 1:25 and 1:20 respectively. This showed a mild cell toxicity of these products to the PBMCs. Comparatively, the viability concentration of the PRO 2000 to the PBMCs was similar to that of $CaCo_2$ cells. However, the

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viability concentration of the UC781 was two and half time lower at the 1:20 than the 1:50 found in $CaCo_2$ cells. In contrast to the other product and comparable to the $CaCo_2$ cells, the nontoxic concentrations of Vena Gel™ (Figure 2e) and N9 (Figure 2f) were 1:1000. These showed 40- to 100-fold more toxicity to peripheral blood mononuclear cells (PBMC) than the other four products.
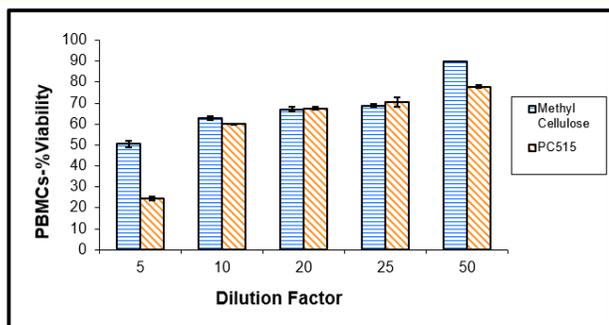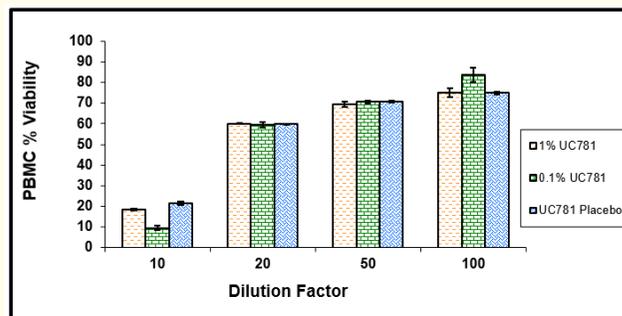

Figure 2a: Comparative peripheral blood mononuclear cells (PBMC) viabilities at the various dilutions of Carraguard™ (PC515) and its Methyl Cellulose placebo.
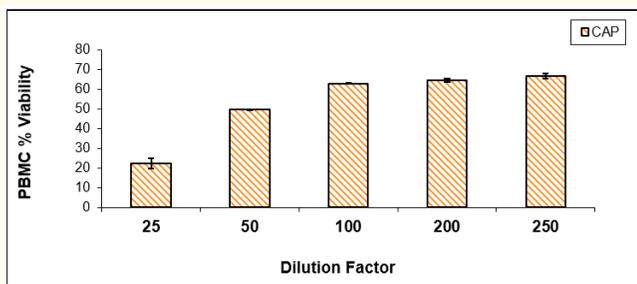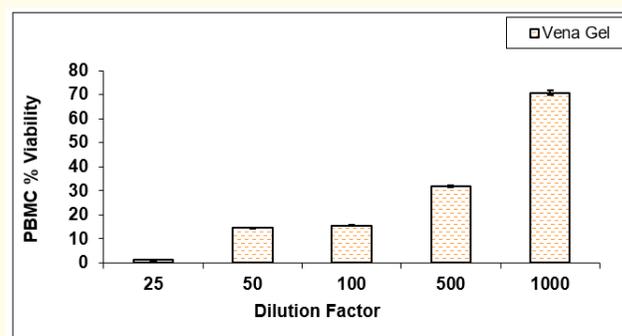

Figure 2b: Comparative peripheral blood mononuclear cells (PBMC) viabilities at the various dilutions of Cellulose Acetate Phthalate (CAP).
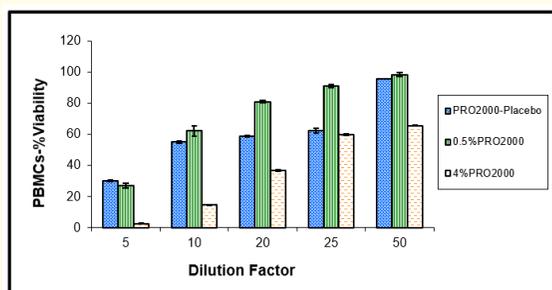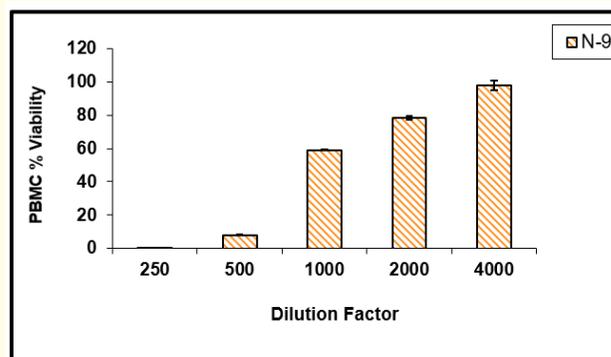

Figure 2c: Comparative peripheral blood mononuclear cells (PBMC) viabilities at the various dilutions of 4% and 0.5% PRO 2000 and the placebo.

Figure 2d: Comparative peripheral blood mononuclear cells (PBMC) viabilities at the various dilutions of 1% and 0.1% UC781 and the placebo.

Figure 2e: Comparative peripheral blood mononuclear cells (PBMC) viabilities at the various dilutions of VenaGel.


Figure 2f: Comparative peripheral blood mononuclear cells (PBMC) viabilities at the various dilutions of KY Jelly and KY-N9.

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The nontoxic dilutions of the five placebos ranged from 1:5 for methyl cellulose to 1:100 for the Vena Gel placebo and KY Jelly. The results showed that 0.5% PRO 2000 product was less toxic to the PBMC cells than the 4% product and the placebo. Furthermore, the nontoxic dilution of the 4% PRO 2000 and that of the UC781 formulations were equal to their placebos. This further confirms that most of the toxicities in these products were due to the excipients. In contrast, the nontoxic dilutions of N9 and Vena Gel™ were 10 times greater than those of their respective placebo formulations as in the $CaCo_2$ cells. This is a further indication of the toxicities of active drug ingredients these products rather than the base formulations or formulation vehicles. No significant change in toxicity occurred when nontoxic dilutions of the products and placebos were applied to epithelial cell cultures for 2-hours each day for 5-days.

Microbicide toxicities to the macrophages

The products toxicities to macrophages followed a similar pattern to that of the $CaCo_2$ cells and the peripheral blood mononuclear cells (PBMCs) (Table 1; Figure 3). The nontoxic or cell viability concentration of the Carraguard™ (PC515) (Figure 3a) to the macrophages was 1:5. This was similar to that of the $CaCo_2$ cells but two times less than that of the PBMCs of 1:10. The nontoxic or cell viability concentration of the cellulose acetate phthalate (CAP) (Figure 3b) to the was macrophages was 1:50. This concentration is two times more than to the $CaCo_2$ cells (Table 1) but half of that of the PBMCs. The nontoxic concentrations of the PRO 2000 (0.5% and 4% (Figure 3c) and UC781 (0.1% and1%) (Figure 3d) were all 1:20. The nontoxic concentration of the PRO 2000 were slightly lower that the 1:25 found in $CaCo_2$ cells and PBMC.
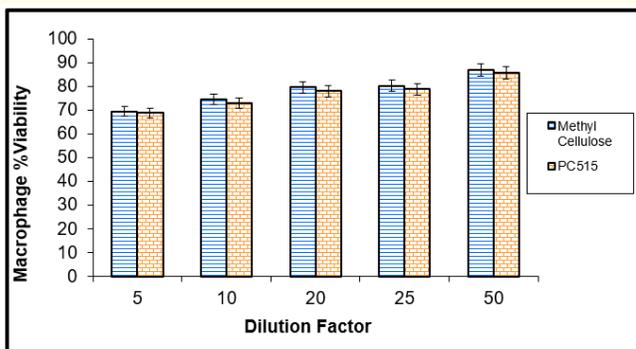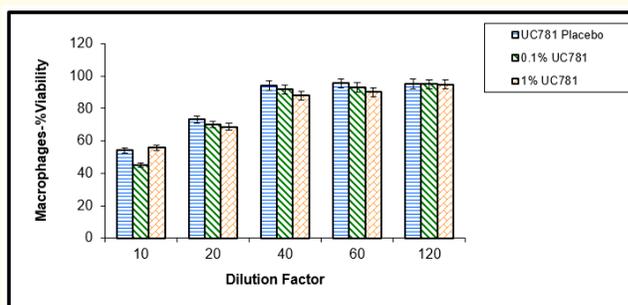

Figure 3a: Comparative Macrophage cell viabilities at the various dilutions of Carraguard™ (PC515) and its Methyl Cellulose placebo.
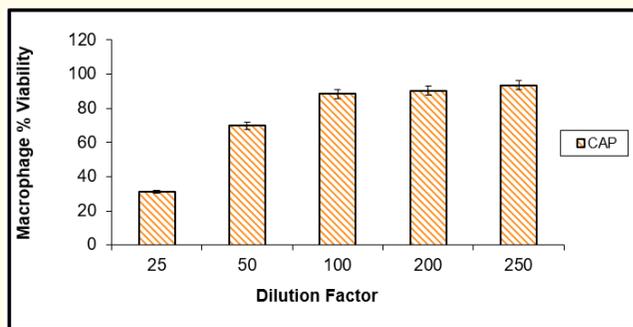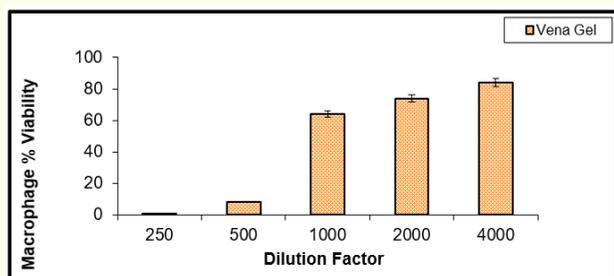

Figure 3b: Comparative macrophage cell viabilities at the various dilutions of Cellulose Acetate Phthalate (CAP).
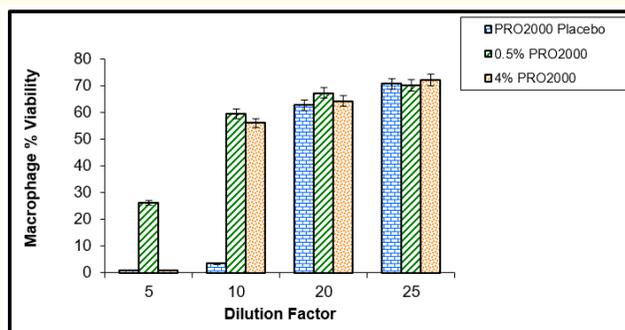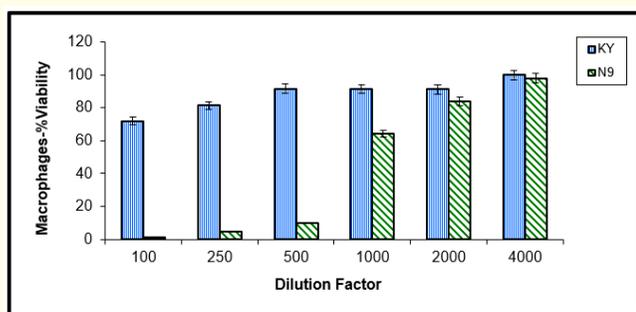

Figure 3c: Comparative macrophage cell viabilities at the various dilutions of 4% and 0.5% PRO 2000 and the placebo.

Figure 3d: Comparative macrophage cell viabilities at the various dilutions of 1% and 0.1% UC781and the placebo.

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Figure 3e: Comparative macrophage cell viabilities at the various dilutions of VenaGel.

Figure 3f: Comparative macrophage cell viabilities at the various dilutions of KY Jelly and KY-N9.

The nontoxic concentration of the UC781 to the macrophages was the same as that of the PBMC. Thus, the UC781 was two and half times less toxic to the immune cells than the $CaCo_2$ epithelial cells. Strikingly, the nontoxic or cell viability concentrations of the placebos of the Carraguard™ (PC515), PRO 2000 and UC781 were like those of all the drug concentrations of the products. This confirms toxicities of these product excipients. As in the $CaCo_2$ cells and the PBMCs, the nontoxic concentrations of Vena Gel™ (Figure 3e) and N9 (Figure 3f) products were 1:1000 and the placebo were similarly at 1:100. These means that the two products were up to 100-fold more toxic to all immune cells and epithelial cells than the four other products, and that the toxicities were from the active drug products than their base formulations or formulation vehicles.

Proinflammatory cytokine release

Many investigators including Belec and co-workers have the content of proinflammatory cytokines in vaginal secretions of non-HIV infected women in picograms per milliliter reported as between 310 - 960 for IL-1β, 75 - 510 for IL-6 and 30 - 210 for TNF-α. The ranges for HIV infected women at different stages of

infection ranged from 375 - 2800 for IL-1β, 190 - 2160 for IL-6 and 53 - 570 for TNF-α [16].

In this project, CAP showed no toxicity in terms of proinflammatory cytokine release in PBMCs when treated with non-toxic dilutions. For example, IL-1β, TNF-α, IL-6 and IL-8, levels were 1.305, 0.175, 0.195 and 8.75pg/ml, respectively (figures 4, 6, 8, and 10). Similarly, when treated with toxic dilutions IL-1β, TNF-α, IL-6 and Il-8, levels were 1.767, 1.815,1.94 and 22.50 pg/ml respectively. Apart from IL-1β and as expected, there were statistically significant differences between the toxic and non-toxic dilutions (p < 0.001). CAP also, showed similar results with no toxicity in terms of cytokine levels in macrophages when treated with nontoxic dilutions. For example, IL- 1β, TNF-α, IL-6 and Il-8, levels were 0.815, 0.111, 0.175 and 31.88 pg/ml, respectively (Figures 5,7, and 9). Similarly, when treated with toxic dilutions of CAP, IL-1β, TNF-α, IL-6 and Il-8, levels were 0.776 1.815, 1.112 and 69.59 pg/ml, respectively. Apart from IL-1β and as expected, there were statistically significant differences between the toxic and non-toxic dilutions (p < 0.001).

Nonoxynol-9 and KY jelly showed no toxicity in terms of cytokine levels in PBMCs and macrophage after being treated with both toxic and nontoxic dilutions. The values of IL-1 β, TNF-α, were 0.1 - <2.0 pg/ml for both PBMCs and macrophages. The levels of IL-8 ranged 9 - 13.00 pg/ml and 12 - 16 pg/ml for PBMCs and macrophages, respectively (Figures 10 and 11). The results were not statistically significant for IL1 β, IL-6 and IL-8 (p > 0.05) when N9 was compared to KY-jelly. However, the results were significantly different when comparing N9 to KY-jelly for TNF-α (p < 0.001).
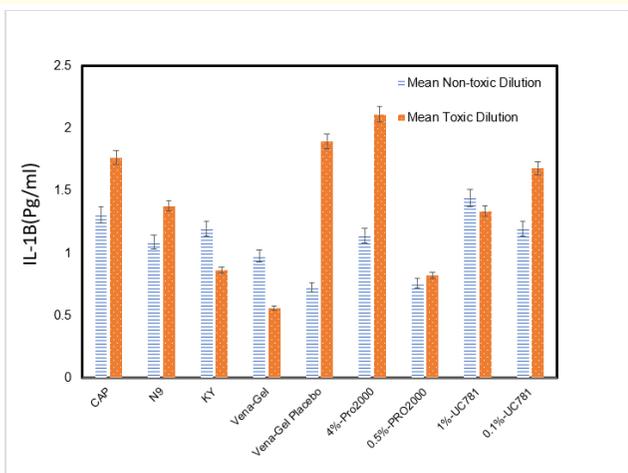
Vena Gel and vena Gel placebo showed no toxicity in terms of cytokine levels in PBMCs and macrophages when treated with both toxic and nontoxic dilutions. The value of Il-1 β, TNF-α ranged from 0.1 - 3.0 pg/ml for both PBMCs and macrophages. The levels of IL-8 ranged from 10 - 61.25pg/ml and 119 - 303 pg/ml for PBMCs and macrophages, respectively (Figures 10 and 11).

Pro2000 (4% and 0.5%) showed no toxic levels of cytokines in PBMCs and macrophages when treated with both toxic and nontoxic dilutions. The concentration levels of IL-1β, TNF-α, and IL-6 were 0.1 - 0.2 pg/ml and 0.6 - 4.0 pg/ml for PBMCs and macrophages respectively. The levels of IL-8 were 13 pg/ml for PBMCs when exposed to 4% and 0.5% PRO2000 (Figure 10). Conversely, the concentration of IL-8 for macrophages ranged between 43 - 70 pg/ml for both formulations (4% and 0.5%) (figure 11). The results
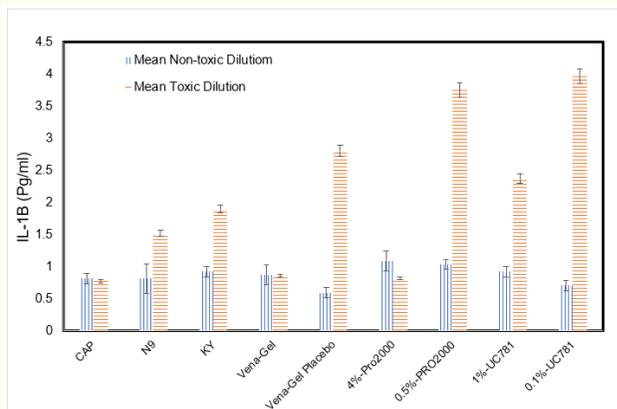
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were not statistically significant for IL 1β, IL-6 and IL-8 (p > 0.001) when comparing the two formulations. However, statistically significant differences were observed for TNF-α (p < 0.001).

UC781 (1% and 0.1%) showed no toxic level of cytokines in PBMCs and macrophage when treated with both toxic and non-toxic dilutions. The concentrations of IL-1β, TNF-α, and IL-6 were 0.01 - 2.0 pg/ml and 0.2 - 4.0 pg/ml for PBMCs (Figures 4, 6, 8 and 10) and macrophages (Figures 5, 7, 9 and 11), respectively. The levels of IL-8 were < 22 pg/ml for PBMCs when exposed to 1% and 0.1% UC781. Conversely, the concentration of IL-8 in macrophages range between 18-50 pg/ml for both formulations (1% and 0.1%). The results were not statistically significant for IL-1β, IL-6 (p > 0.001) when comparing the two formulations. Yet, statistical significance was observed for TNF-α AND IL-8 (p < 0.001).

In general, these increases in concentrations of these proinflammatory cytokines from the microbicide products in these immune cells were insignificant compared to the normal contents in normal vaginal secretions of between 310 - 960 for IL-1β, 75 - 510 for IL-6 and 30 - 210 for TNF-α. The ranges for HIV infected women at different stages of infection ranged from 375 - 2800 for IL-1β, 190 - 2160 for IL-6 and 53 - 570 for TNF-α [16].

Figure 5: The effect of the microbicide formulation on IL-1B in primary human macrophages. Macrophages were suspended in 1 ml media and mixed with toxic and nontoxic formulations of microbicides for 4 hrs. The products were tested in triplicate. Post exposure to microbicides, the supernatants were collected and assayed for cytokine IL-1B release using ELISA (R & D systems). The data present are averages ± standard deviations of the means.
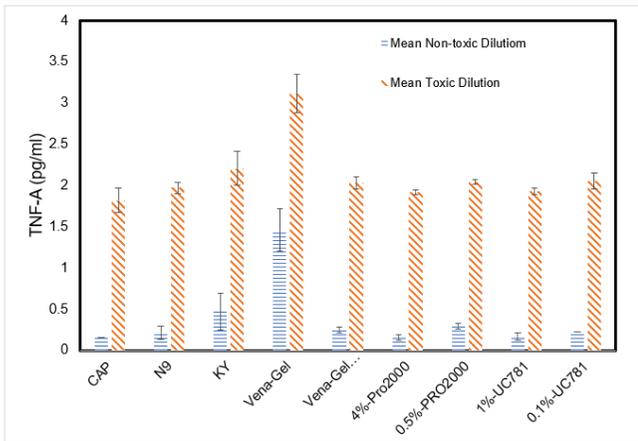
Figure 4: The effect of the microbicide formulation on IL-1Bin peripheral blood mononuclear cells (PBMCs). PBMCs were suspended in 1 ml media and mixed with toxic and nontoxic formulations of microbicides for 4 hrs. The products were tested in triplicate. Post exposure to microbicides, the supernatants were collected and assayed for cytokine IL-1B release using ELISA (R & D systems). The data present are averages ± standard deviations of the means.
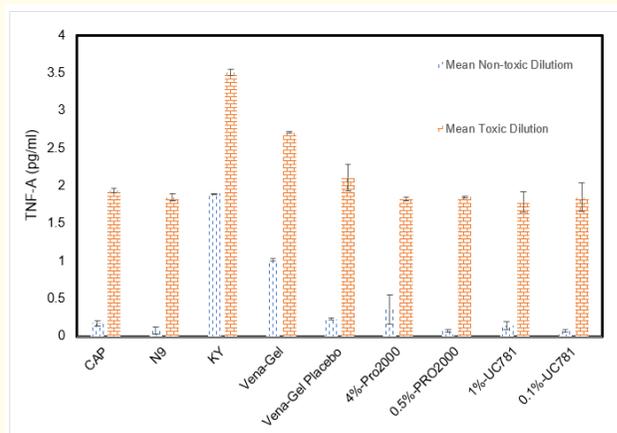
Figure 6: The effect of the microbicide formulation on pro-inflammatory TNF-α in peripheral blood mononuclear cells (PBMCs). PBMCs were suspended in 1 ml media and mixed with toxic and nontoxic formulations of microbicides for 4 hrs. The products were tested in triplicate. Post exposure to microbicides, the supernatants were collected and assayed for cytokine TNF-α release using ELISA (R & D systems). The data present are averages± standard deviations of the means.

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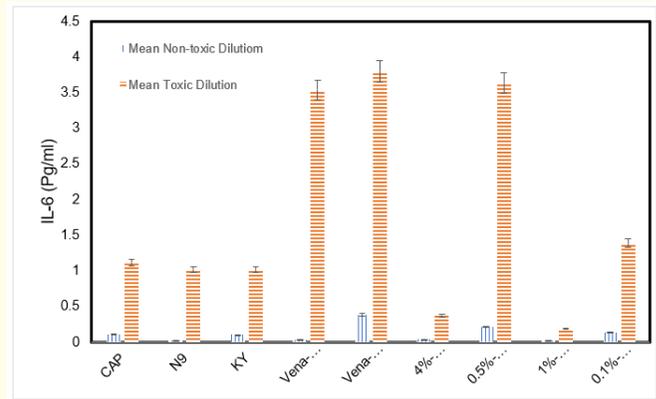**Figure 7:** The effect of the microbicide formulation on TNF-α in primary human macrophages. Macrophages were suspended in 1 ml media and mixed with toxic and nontoxic formulations of microbicides for 4 hrs. The products were tested in triplicate. Post exposure to microbicides, the supernatants were collected and assayed for cytokine TNF-α release using ELISA (R & D systems). The data present are averages± standard deviations of the means.

Figure 9: The effect of the microbicide formulation on pro-inflammatory cytokine IL-6 in primary human macrophages. Macrophages were suspended in 1 ml media and mixed with toxic and nontoxic formulations of microbicides for 4 hrs. The products were tested in triplicate. Post exposure to microbicides, the supernatants were collected and assayed for cytokine IL-6 release using ELISA (R & D systems). The data present are averages± standard deviations of the means.

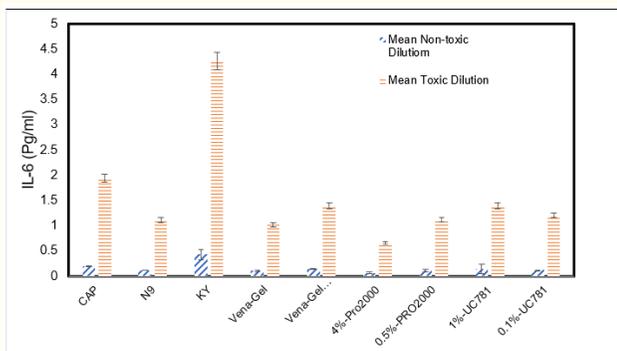**Figure 8:** The effect of the microbicide formulation on pro-inflammatory cytokine IL-6 in peripheral blood mononuclear cells (PBMCs). PBMCs were suspended in 1 ml media and mixed with toxic and nontoxic formulations of microbicides for 4 hrs. The products were tested in triplicate. Post exposure to microbicides, the supernatants were collected and assayed for cytokine IL-6 release using ELISA (R & D systems). The data present are averages± standard deviations of the means. ±

Figure 10: The effect of the microbicide formulation on IL-8 levels in peripheral blood mononuclear cells (PBMCs). PBMCs were suspended in 1 ml media and mixed with toxic and snontoxic formulations of microbicides for 4 hrs. The products were tested in triplicate. Post exposure to microbicides, the supernatants were collected and assayed for cytokine IL-8 release using ELISA (R & D systems). The data present are averages± standard deviations of the means.

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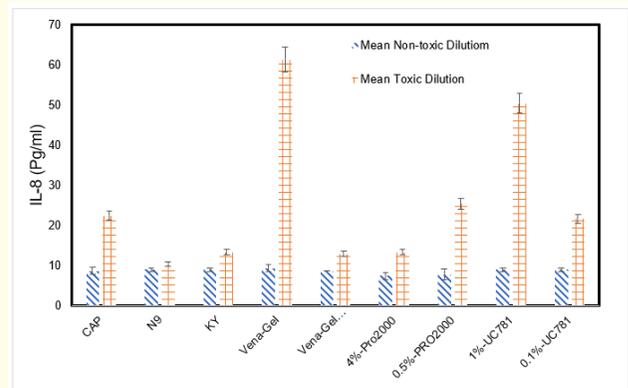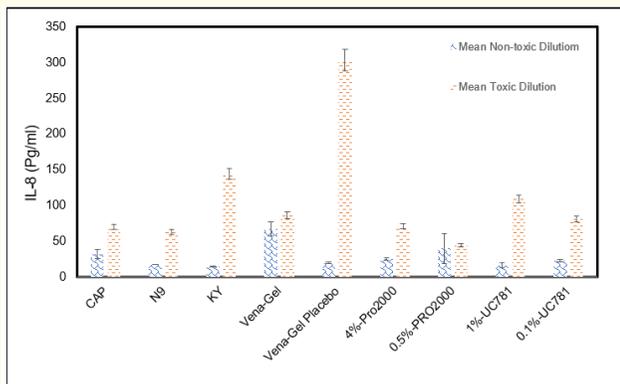
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Figure 11: The effect of the microbicide formulation on IL-8 levels in primary human macrophages. Macrophages were suspended in 1 ml media and mixed with toxic and nontoxic formulations of microbicides for 4 hrs. The products were tested in triplicate. Post exposure to microbicides, the supernatants were collected and assayed for cytokine IL-6 release using ELISA (R & D systems). The data present are averages ± standard deviations of the means.

Discussion

The data from the project showed that CAP, PRO 2000 (0.5% and 4%), and UC781 (0.1% and 1%) were relatively nontoxic against $CaCo_2$ epithelial cells and primary immune cells of PBMCs and Macrophages. The KY-N9 and Vena Gel™ formulations were much more toxic with high nontoxic or cell viability product dilutions against the $CaCo_2$ epithelial cells and primary immune cells PBMCs and Macrophages. Nonoxymol-9 was originally developed as a spermicide but initial reports showed it had *in vitro* activity against sexually transmitted diseases including HIV-1 [17,18]. Despite this promising early work, it has been reported that the N9 antiviral activity only occurred at doses that were cytotoxic [19]. Several clinical studies have reported epithelial disruption and inflammation of the female genital tract [10,20,21]. and two additional studies showed toxicity of the rectal mucosa in humans and nonhuman primates [22,23]. Since that time, the recommendation has been that products containing N-9 should not be used for HIV-1 prevention [6]. Our data were consistent with these results showing that KY-N9 was highly toxic to the cells and the nontoxic concentrations were too dilute to be effective. Likewise, the 1% Vena Gel™ preparation was highly

toxic at its original concentration. This was unexpected since other antibiotic peptides, such as Defensins, are used at concentrations approximately 1000-fold higher [24]. Moreover, animal studies showed mild vaginal irritation at the 1% concentration and greater vaginal irritation at higher concentrations. Both CAP and Carraguard® are used in the pharmaceutical industry and are classified as "generally recognized as safe" compounds. The animal vaginal irritation studies showed that CAP did not have any deleterious effects. Preliminary safety and acceptability trials have showed carrageenan products to be well tolerated in humans [27]. UC781 have shown low toxicity in preclinical studies and PRO 2000 has shown low toxicity in preclinical and phase I clinical studies [26,27]. The comparison evaluation in this study for toxicity of the microbicide formulations was consistent with the findings from these published and suggests that our evaluation was predictive.

Inflammation of the female reproductive tract increase susceptibility to HIV-1 and other viral infections and, thus, it becomes a serious liability for vaginal products. Excessive release of proinflammatory cytokines may alter the mucosal balance between tissue destruction and repair and be linked to enhanced penetration and replication of viral pathogens upon chemical insult. In this study, emphasis was placed on the value of interleukin (IL)-1 β, TNF-α, IL-6, AND IL-8 as markers of mucosal toxicity because they represent families of cytokines with different roles in inflammatory tissue damage and HIV-1 pathogenesis. IL-1β, TNF-α and IL-6 induce HIV-1 expression via NF-κB mediated HIV-1 long terminal repeat (LTR) activation, while IL-8 triggers the recruitment of HIV-1-susceptible cells to the inflammation site and may also stimulate HIV-1 replication in T cells and macrophages [28]. All these cytokines are expressed constitutively at low levels by cervicovaginal keratinocytes and are significantly induced via NF-κB mediated signal transduction pathways by pro-inflammatory stimuli and common sexually transmitted pathogens [28].

In this study, the correlation between the response of peripheral blood mononuclear cells (PBMC) and macrophages from non-HIV-infected cells was investigated. As a result, PBMCs and macrophages were divided into 2 groups according to the microbicides dilutions. Higher concentrations of proinflammatory cytokines in genital secretions have been associated with higher levels of vaginal virus in HIV-1- infected women. Therefore, it was be important to know if non-toxic levels of the microbicides increase production of these cytokines [16].

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Currently, there is no commercially available microbicide with anti-HIV activity. However, it is a pivotal time in HIV microbicide research, wherein more resources are becoming available and progress is being made in the field. For example, the well-known spermicide Nonoxynol-9 (N9) that had been shown to inactivate HIV-1, N-9 actually increases the risk of HIV-1 transmission [8], due to increase HIV-1 shedding and increased recruitment of CD4+ target cell associated with vaginal inflammation [29] indicated that N-9 use promote HIV-1 transmission through Interleukin-mediated NF-κB activation, and increased replication.

In this study, N-9 showed low levels of TNF-α, IL-1β and IL-6 cytokines were <2 pg/ml, whereas elevated level of IL-8 was observed, showed that N-9 induced IL-α/β release, activation NF-κB and subsequent IL-8 up-regulation by cervical and vaginal epithelial in *in vitro* [29]. Ayehunie and colleagues [30] also showed the effects of formulations containing the common spermicide active ingredient, nonoxynol-9 (N9), on epithelial tissue containing epithelial VEC cells, with increasing N9 concentration, the tissue viability decreases and the IL-1β release increases. Such assays can be used to predict toxicity of vaginal care products, microbicides, and other chemical agents.

Reports indicate that cellulose acetate phthalate (CAP) not only inactivate HIV, HSV-1, HSV-2, and several other microbes *in vitro*, but that it does not affect lactobacilli, part of the natural vaginal flora which helps resisting STDs [31]. Also, our data indicated that CAP has shown nontoxicity in terms of cytokines release in our two cellular models. While sustaining stability and anti-HIV-1 activity in the epithelial environment, CAP did not increase the production of proinflammatory mediators during or after exposure, nor did it modify the epithelial resistance to leukocyte traffic [31]. CAP attenuated some TNF-α-induced responses but did not interfere with epithelial cytokine responsiveness to gonococcal determinants. The described system may be useful for predicting proinflammatory side effects of other microbicide candidates for vaginal application.

Other candidate vaginal microbicides studied were PRO2000 (4% and 0.5%), Vena Gel and Placebo [4], and reports indicated that PRO2000 has *in vitro* activity against HIV-1 strains, as well as having low epithelial cellular toxicity. Our data indicated that PRO2000 did not enhance the release of cytokine levels significantly. The data also indicated that PC515 and UC 781 (1% and 0.1%) showed very low production of cytokines in both PBMCs and Macrophages. These finding supported the clinical trials results utilizing carraguard in HIV-1 infected study in South Africa and Thailand [32]. The data has indicated that microbicides induced relatively higher levels of IL-8 in PBMCs and macrophages. The disadvantage is that IL-8 might triggers the recruitment of HIV-1-susceptible cells to the inflammation site and may also stimulate HIV-1 replication in T cells and macrophages [28].

Research reports have indicated that many factors are associated with vaginal inflammation such as IL-1β, IL-6 and TNF-α which are found in mucosal secretions of chronically infected HIV-patients [16]. Each of these factors is reported to enhance HIV-1 replication *in vitro*, and their presence in cervicovaginal secretions may have enhanced HIV-1 replication in the genital tract of the women [33].

Conclusion

All the microbicides tested in their toxic and non-toxic formulation in the two models of PBMCs and macrophages showed relatively low levels of IL-1β, TNF-α and IL-6. This indicated safety and low toxicity of these microbicides in terms of cytokine release. Also, it showed that usage of these microbicides as anti-HIV formulation will not trigger cytokine release. On the other hand, IL-8 has shown relatively higher levels for all microbicides tested in toxic and nontoxic formulation. This indicated that microbicides induced the release of IL-8 and could trigger the recruitment of HIV-1 susceptible cells and increase HIV-1 replication. The only way to support these findings is through clinical trials phase-I safety and acceptability studies.

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