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The Trichloroethylene Metabolite *S*-(1,2-dichlorovinyl)-L-cysteine but not Trichloroacetate Inhibits Pathogen-Stimulated TNF- α in Human Extraplacental Membranes In Vitro

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Abstract

Extraplacental membranes define the gestational compartment and provide a barrier to infectious microorganisms ascending the gravid female reproductive tract. We tested the hypothesis that bioactive metabolites of trichloroethylene (TCE) decrease pathogen-stimulated innate immune response of extraplacental membranes. Extraplacental membranes were cultured for 4, 8, and 24 h with the TCE metabolites trichloroacetate (TCA) or *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) in the absence or presence of lipoteichoic acid (LTA) or lipopolysaccharide (LPS) to simulate infection. In addition, membranes were cocultured with DCVC and Group B *Streptococcus* (GBS). DCVC (5–50 μ M) significantly inhibited LTA-, LPS-, and GBS-stimulated cytokine release from tissue punch cultures as early as 4 h ($p < 0.05$). In contrast, TCA (up to 500 μ M) did not inhibit LTA-stimulated cytokine release from tissue punches. Because cytokines are important mediators for host response to infectious microorganisms these findings suggest that TCE exposure could potentially modify susceptibility to infection during pregnancy.

Keywords

Trichloroethylene; toxicant-pathogen interactions; pregnancy; Group B *Streptococcus*; dichlorovinyl cysteine

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1. Introduction

The primary pathway of intrauterine infection is via the ascending pathway, progressing from the vagina and across the extraplacental membranes to enter the amniotic cavity [1, 2]. Other pathways through maternal-fetal blood exchange in the placenta contribute to intrauterine infection to a lesser extent. Thus, the extraplacental membranes (also known as gestational and maternal-fetal membranes) form a critical barrier to infection of the gestational compartment. The innate immune response includes cytokines and antimicrobial peptides that are important components of host defense against microbial infection.

Although intrauterine infection, including infection of the extraplacental membranes, is recognized as a leading etiological factor associated with adverse birth outcomes [1, 3-6], the ability of common environmental contaminants to modify susceptibility of the female reproductive tract to infection during pregnancy has scarcely been explored. Indeed, recent articles have highlighted the need for increased research on toxicant-pathogen interactions, in general [7-9]. Given the likelihood that pregnant women are exposed to pollutants in their workplaces, homes, and outdoor environment, limited knowledge of toxicant actions on host defense of extraplacental membranes to infection is a critical barrier to understanding the mechanisms of adverse pregnancy outcomes.

Trichloroethylene (TCE) is a colorless volatile industrial solvent primarily used for metal degreasing. TCE is also a widespread environmental contaminant of concern, ranked #16 on the Agency for Toxic Substances and Disease Registry (ATSDR) 2011 Priority List of Hazardous Substances [10] and recently reclassified as a known human carcinogen by the US Environmental Protection Agency (EPA) [11]. Recent reports show increased mortality, decreased bacterial clearance, and decreased alveolar phagocytosis in rodents co-treated with TCE and *Streptococcus zooepidemicus* [12, 13]. Metabolism appears to be necessary for at least some of TCE's immunomodulatory activity *in vivo* [14]. Most absorbed TCE is rapidly metabolized via one of two irreversible pathways [15]. The first pathway involves cytochrome P450-mediated oxidation, with trichloroacetic acid (TCA) and dichloroacetic acid (DCA) as the major bioactive metabolites [16]. The second TCE metabolic pathway involves conjugation with glutathione to form *S*-(1,2)-dichlorovinyl glutathione (DCVG), which is further metabolized to the bioactive metabolite *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) [17]. TCA and DCVC have been detected in serum of exposed rodents [16, 18, 19] and humans [20]. The placenta is a highly perfused organ that would readily be exposed to circulating TCE and metabolites. In addition, the human placenta expresses key enzymes necessary to initiate TCE metabolism to bioactive forms, including CYP2E1, which is important for TCA formation [21, 22] and glutathione-*S*-transferase (GST) [23], needed to generate DCVC. Here we utilized human extraplacental membranes cocultured with LTA, LPS, and GBS to test our hypothesis that trichloroethylene, through its metabolites, decreases the innate immune response of extraplacental membranes. LTA and LPS were used as model infectious agents because they are known to induce inflammatory responses in the extraplacental membranes [24, 25]. In addition we utilized live GBS as a model bacterium because it remains the leading cause of infectious neonatal morbidity and mortality in the US [26].

2. Material and Methods

2.1. Reagents and Materials

TCA (purity 99%) was from Sigma-Aldrich (St. Lewis, MO). DCVC was synthesized at the University of Michigan Medicinal Chemistry Core Synthesis Lab according to procedures described by McKinney et al. [27]. Purity (98.7%) was determined by HPLC analysis and identity was confirmed by proton nuclear magnetic resonance spectroscopy. The Group B *Streptococcus* (GBS; *S. agalactiae*) strain used in this study (A909, construct RS020, a gift from Amanda Jones, University of Washington), was initially isolated from a septic newborn [28]. GBS was grown at 37 °C in culture using Todd Hewitt Broth (THB, Becton-Dickinson, Franklin Lakes, NJ) or on sheep's blood agar plates (Blood Agar Base #2, Remel, Lenexa, KS and BBL defibrinated sheep blood, Franklin Lakes, NJ) with 5 µg/mL erythromycin (Hemostat Labs, Dixon, CA). Media, buffers, fetal bovine serum (FBS; catalog #10438), 0.25% trypsin-EDTA (catalog #25200), and penicillin/streptomycin (pen/strep; catalog #15140) were from GIBCO (Grand Island, NY). Lipoteichoic acid (LTA) from *Staphylococcus aureus* was from Invivogen (San Diego, CA). Lipopolysaccharide (LPS) from *Salmonella typhimurium* was from List Biological Laboratories (Campbell, CA).

2.2. Culture of Extraplacental Choriodecidual Membranes

As previously described, human extraplacental membranes were collected from healthy, non-laboring women undergoing scheduled cesarean delivery at the University of Michigan Von Voigtlander Women's Hospital Birth Center [29]. This research was approved by University of Michigan Institutional Review Board (IRBMED #HUM0013915).

Upon delivery, the membranes were transported to the research laboratory in Dulbecco's phosphate-buffered saline (DPBS). Blood clots were removed by gently massaging the tissue and rinsing with DPBS. Explants of membranes were obtained using a 12-mm biopsy punch and placed in 12-well plates with 1 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% FBS and pen/strep. Pen/strep was used to avoid potential contamination during tissue collection. Cultures were incubated at 37 °C and 5% CO₂. After 4 h, the medium was changed, and cultures were incubated an additional 24 h. LTA and LPS were initially dissolved in sterile molecular grade water, and then diluted in DMEM/1% FBS/pen/strep without or with LTA or LPS to make exposure medium immediately prior to addition to cultures. Following the 24-h tissue acclimation, the medium of the extraplacental membranes punches was replaced with exposure medium with the following treatments: medium only (control), LTA (1 µg/mL), LPS (100 ng/mL), DCVC (1, 5, 10, 20, or 50 µM), TCA (10, 50, 100 or 500 µM), DCVC (1, 5, 10, 20, or 50 µM) + LTA (1 µg/mL), DCVC (10, 20 or 50 µM) + LPS (100 ng/mL), or TCA (50, 100 or 500 µM) + LTA (1 µg/mL). Tissue punches were incubated with treatments for 4, 8, or 24 h. Treatments were balanced across donors with three replicates for each treatment using extraplacental membranes from a minimum of five women.

Next, to better simulate ascending uterine infection, extraplacental membranes were cultured with live GBS in a two-compartment transwell system as described previously [24, 30]. Briefly, extraplacental membranes were secured to transwell frames lacking synthetic

membranes (gift from Corning, NY) using latex elastic bands (ORMCO, Orange, CA), with the choriodecidual side of the extraplacental membranes facing the inside of the transwell insert. The transwell inserts with mounted tissue were placed in wells of 12-well culture plates containing DMEM/1% FBS/pen/strep medium. To maintain equal medium levels across the transwell chambers, there was 0.5 mL medium in the inner chamber and 1.5 mL medium in the outer chamber. Cultures were incubated at 37 °C and 5% CO₂ for 4 h, and then the medium was exchanged with medium without antibiotics. GBS was grown in Todd Hewitt Broth, isolated in early exponential growth phase, and diluted in tissue culture medium without antibiotics to 1×10⁶ colony forming units/mL (CFU/mL). Following 24-h incubation, the medium in the inner choriodecidual-facing chamber was replaced with fresh DMEM/1%FBS exposure medium containing treatment combinations of GBS (1×10⁶ CFU/mL) and DCVC: 0.5 mL GBS inoculant (1×10⁶ CFU/mL), DCVC (5 or 10 μM), or DCVC (5 or 10 μM) + GBS (1×10⁶ CFU/mL). Medium in the outer amnion-facing compartment was replaced with fresh DMEM/1%FBS containing DCVC (5 or 10 μM). Control cultures had fresh medium without DCVC or GBS. Cultures were incubated with treatment for 4, 8, or 24 h. Transwell experiments were conducted in duplicate or triplicate using extraplacental membranes from five women with treatments balanced across women.

2.3.Cytokine ELISAs

Cytokine ELISAs on extraplacental membranes medium were performed by the University of Michigan Immunology Core using commercially available enzyme linked immunosorbant assays (ELISA; R&D Systems). The ELISA detection ranged from 2.91-2500 pg/mL for IL-1β, 9.38-125,000 pg/mL for IL-6, 31.2-2000 pg/mL for IL-8, and 15.6-5000 pg/mL for TNF-α. Samples were diluted as necessary and values are reported as pg or ng protein/mL medium. Samples below the limit of detection (LOD) were transformed to LOD/ 2 prior to statistical analysis [31].

2.4.RNA Extraction and PCR

After culture with treatments, punches (12 mm) of extraplacental membranes were homogenized in RIPA buffer with Lysing matrix D beads using 2-40 s pulses with a 5 min rest on ice on the FastPrep instrument (MP Biosciences). Total RNA from punches was then extracted using RNeasy Plus Mini kits (Qiagen, Valencia, CA). The cDNA was synthesized with 1 μg total RNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA) according to manufacturer's instructions. Three replicates per treatment within each woman were pooled. Forward and reverse primers used were: (TNF-α) 5'-GGAGGACGAACATCCAACCTT-3' and 5'-GGTTGAGGGTGTCTGAAGGAG; beta-2-microglobulin (B2M) 5'-TGGAGGCTATCCAGCGTACT-3' and 5'-CGGATGGATGAAACCCAGACA-3'. Quantitative RT-PCR reactions were performed on a Bio-Rad CFX Connect Real-Time System according to SsoAdvanced SYbR Green Supermix directions (BioRad, Hercules, CA). The following conditions were used for PCR: 95 °C for 10 min, followed by 40 cycles of 15 sec at 95 °C, 5 sec at 60 °C. Signal intensities of target genes were quantified using standard curves and normalized to the signal of β-microglobulin using Bio-Rad CFX manager software. All pooled samples from above were run in triplicate. Data were analyzed using CFX software (BioRad, Hercules, CA).

2.5.GBS Viability

GBS viability in the presence of DCVC and TCA was assessed using the AlamarBlue assay (Serotec) according to manufacture directions and previously published methods [32]. Briefly, GBS was grown to exponential log phase at 37 °C with shaking in THB and diluted to 1×10^6 CFU/mL in DMEM medium supplemented with 1% FBS. Bacterial suspension and AlamarBlue dye (10%) were incubated with DCVC or TCA in 96-well plates at 37 °C. Fluorescence was measured after 8 h on a SpectraMax M2^e plate reader (excitation 560 nm, emission 590 nm), and percent cell viability was determined. Three independent experiments were performed with five replicates per treatment.

2.6. Statistical Analysis

Values were averaged for replicates within each woman. Data are presented as mean $\pm$ SEM and were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). ANOVAs with Tukey's post hoc test were performed. Data were considered significant if the p-value was < 0.05 .

3. Results

3.1. DCVC Effects on LTA-Stimulated and LPS-Stimulated TNF- α Release from Tissue Punch Cultures of Extraplacental Membranes

To investigate TCE metabolite-pathogen interactions on cytokine release, human extraplacental membranes were cocultured with the TCE metabolites TCA or DCVC in the absence or presence of LTA (1 μ g/mL) or LPS (100 ng/mL) for 24 h. The LTA and LPS concentrations were selected because they were previously shown to stimulate cytokines in the extraplacental membranes tissue model [33]. Exposure to TCA or DCVC alone did not significantly increase cytokine release from extraplacental membranes, whereas LTA and LPS each significantly increased TNF- α release from control levels of approximately 20 pg/mL to 830 pg/mL and 4300 pg/mL respectively (Supplemental Figure 1; $P < 0.05$). With co-exposure, however, 5 μ M DCVC significantly inhibited LTA-stimulated TNF- α release by 5 fold. In addition, 10 μ M DCVC (the lowest concentration tested) significantly inhibited LPS-stimulated TNF- α release with a 21-fold reduction (Supplemental Figure 1). To investigate the toxicodynamics of DCVC inhibition of pathogen-stimulated TNF- α release, extraplacental membranes were exposed to DCVC alone or co-exposed to DCVC and LTA for 4, 8, and 24 h. LTA stimulated a robust increase in TNF- α by 4 h which was sustained at 8 and 24 h. Co-exposure to 10 μ M DCVC inhibited the LTA-stimulated response at 4, 8, and 24 h (Figure 1; $P < 0.05$). To further probe the mechanism by which DCVC inhibited pathogen-stimulated TNF- α release, RNA was extracted from tissue punches treated for 4 h with 10 μ M DCVC, LTA, LTA + 10 μ M DCVC, or no treatment (control). Similar to the protein results, LTA-stimulated TNF- α mRNA expression was inhibited by 4 h of exposure to DCVC by approximately 8 fold (Figure 2; $P < 0.05$). In contrast, the TCE metabolite TCA did not inhibit LTA-stimulated cytokines (Figure 3) at up to 500 μ M.

3.2. DCVC Effects on GBS-Stimulated Release of Pro-Inflammatory Cytokines from Extraplacental Membranes in Transwell Cultures

Extraplacental membranes in transwell culture released increased amounts of IL-1 β (66 fold), IL-8 (4.7 fold), and TNF- α (54 fold) into the medium of the choriodecidual compartment after 24 h of exposure to GBS (Figure 4; $P < 0.05$). GBS exposure did not statistically increase IL-6 release. In addition, GBS-stimulated IL-1 β , IL-8, and TNF- α release were completely inhibited by coculture with 5 μ M and 10 μ M DCVC. To determine if decreased cytokines were a result of DCVC or TCA killing the bacteria directly, GBS viability was assessed after incubation in tissue culture medium with DCVC or TCA for 8 h: DCVC decreased cell viability only at 50 μ M, a higher concentration than we used for the punch and transwell tissue culture experiments (Supplemental Figure 2; $P < 0.05$).

4. Discussion

Despite recent attention to toxicant-pathogen interactions in the literature, few prior studies have examined the role of toxicant-pathogen interactions during pregnancy. Here, we show for the first time that the TCE metabolite DCVC is capable of inhibiting pathogen-stimulated cytokine (IL-1 β , IL-8, and TNF- α) release in a concentration-dependent manner. In addition, we show that this occurs both at the protein and mRNA expression level as early as 4 h. Because cytokines are essential for recruitment and function of immune cells during infection, inhibition of cytokine secretion during infection could result in loss of immune cell recruitment and increased severity of infection.

TCE is a common drinking water contaminant [10] implicated in developmental, reproductive and immune system toxicity [11, 34]. Drinking water contaminated with TCE has been associated with increased neural tube defects, oral clefts, and cardiac defects in humans [35]. Moreover, TCE exposure during pregnancy is associated with intrauterine growth restriction (IUGR) [36]. Although infection is associated with adverse birth outcomes, also [1, 4, 37, 38], few studies have explored toxicant-pathogen interactions during pregnancy despite the likelihood that pregnant women are exposed to both environmental contaminants and infectious agents. Our study is among the first to explore toxicant-pathogen interactions in a relevant target tissue - the extraplacental membranes. Although the extraplacental membranes model is useful for exploring mechanisms of toxicity *in vitro*, our findings of DCVC suppression of pathogen-stimulated cytokines, warrant further validation in pregnant animal models.

Studies in humans and mice implicate a role for TCE in some autoimmune diseases [39]. Workers exposed to TCE have altered cytokine levels including increased IL-2, increased IFN- γ , and reduced IL-4 cytokine levels compared to non-exposed workers [40]. Mice exposed to TCE had increased IL-2, TNF- α , and IFN- γ secretion from CD4+ T-cells [41]. TCE also affects the developing immune system in mice with increased delayed type hypersensitivity, decreased B220 cells in the spleen, and increased thymic T cell subpopulations [42]. Despite evidence linking TCE to reproductive and immune outcomes, few if any studies have examined TCE and immune outcomes (such as cytokines) in pregnant women. Our study is one of the first to use a gestational tissue model to examine effects of TCE metabolites on cytokine secretion. In our study, neither DCVC nor TCA

affected proinflammatory cytokine secretion directly: instead, we saw DCVC suppression of pathogen-stimulated cytokine release. Although the aforementioned studies did not report cytokine suppression, the response could have been overlooked without the stimulus of an infectious agent. Our finding that DCVC suppressed pathogen-stimulated innate immune responses is consistent with prior reports that TCE exposure increases susceptibility to bacterial lung infection in mice [12, 13]. In the latter studies, mice co-treated with TCE and *Streptococcus zooepidemicus* show increased mortality, decreased bacterial clearance in the lung, and decreased alveolar phagocytosis. Although the mechanisms for TCE immunosuppression with bacterial infection have not yet been elucidated, the latter mouse studies, along with our data, provide evidence of toxicant-pathogen interactions with trichloroethylene and its metabolites.

Toxicant inhibition of TNF- α may have implications for pathways necessary for fighting infection, especially in macrophages. TNF- α has a diversity of functions and proper regulation throughout gestation is essential [43-45]. TNF- α is expressed in placental villi during normal pregnancy and regulates apoptotic death in villous cytotrophoblasts [46]. Moreover, TNF- α is involved in neutrophil recruitment and macrophage phagocytosis [47, 48]. Suppression of TNF- α inhibits nitric oxide (NO) production and anti-microbial properties in macrophages [49-52].

Although TCE was detected in rat fetuses following maternal exposure to TCE [53], metabolites were utilized in the present study because most TCE is rapidly metabolized after absorption into the body [11, 15]. Furthermore, TCE metabolites are significantly more biologically active than the parent compound [15], and metabolism appears to be necessary for at least some of TCE's immunomodulatory activity [14]. The primary oxidative pathway of TCE metabolism forms TCA, which is considered the major metabolite responsible for hepatotoxicity [54]. TCE and TCA can be further metabolized to dichloroacetic acid (DCA), which is also thought to contribute to toxicity. However, the relative importance of DCA formation and toxicity in humans is still debated. DCA was not tested in this study because preliminary cell culture experiments did not suggest that DCA was involved in the inflammatory pathway (data not shown). However, additional studies are needed to determine the potential role of DCA during infection of the extraplacental membranes. In addition to the oxidative metabolism pathway, TCE can undergo glutathione-dependent metabolism resulting in DCVC. DCVC is a contributor to nephrotoxicity. For DCVC to be cytotoxic, further metabolism by either a cysteine conjugate beta-lyase or flavin-containing monooxygenase (FMO) is necessary. Despite the fact that little information exists on TCE metabolism in the placenta, the placenta and uterine tissue express both β -lyase and FMOs necessary for DCVC conjugation [55, 56]. Additional experiments are required to determine if the effects observed in the present study are due to DCVC or a downstream metabolite of DCVC.

The effective DCVC concentrations for inhibition of LTA, LPS, and GBS stimulation of TNF- α release in the present study were at or near the lowest bioactive concentrations of DCVC reported in kidney cells [57]. The relevance of concentrations of DCVC to human exposure is difficult to establish due to insufficient data [15]. Humans exposed to levels as low as 100 ppm TCE in air transiently exhibit serum levels as high as 50 μ M of the DCVC

precursor DCVG in serum [20], but typical environmental exposures to TCE are <5 ppb in air [11]. Although the concentrations we used are higher than the average person in the United States would encounter through an ambient environmental exposure, they are within occupational exposure levels [10, 58, 59].

LTA and LPS are cell wall or membrane components of gram positive and gram negative bacteria, respectively, that were used in the present study as model infectious agents because they are highly immunogenic in human extraplacental membranes [24, 25] and induce preterm birth in rodents [60, 61]. GBS is a gram positive bacterium that was used in our study because it is associated with adverse pregnancy outcomes [26, 62, 63] in women, induces preterm labor in nonhuman primates [64], and remains the leading cause of infection-related neonatal death and disease in the United States [26]. In the present study we showed that live GBS mounts a robust cytokine release in extraplacental membranes, consistent with our prior report [29]. Furthermore, we show that LTA and LPS mount similar cytokine responses compared to GBS. Despite the differences in molecular structure of the infectious agents used in this study, DCVC inhibition of pathogen-stimulated cytokines was similar between all three infectious agents (LTA, LPS, and GBS), suggesting the observed cytokine inhibition is not pathogen specific.

In summary, we have shown that the TCE metabolite DCVC inhibits pathogen-stimulated release of pro-inflammatory cytokines IL-1 β , IL-8, and TNF- α . These findings have potential implications for environmental impacts on innate immune response during pregnancy, potentially leading to increased susceptibility to infection. Future studies will focus on additional aspects of immune function such as macrophage function and antimicrobial peptide response. Current limited knowledge of mechanisms by which bacteria act on the host immune system during pregnancy is a critical barrier to the development of targeted preventive and therapeutic approaches to reduce adverse pregnancy outcomes. Findings from this study have the potential to expand current paradigms about GBS infections and environmental contaminant exposures that may put pregnant women at increased risk for intrauterine infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- DCVC inhibited pathogen stimulated cytokine release from tissue punch cultures.
- TCA did not inhibit LTA-stimulated cytokine release from tissue punches.
- TCE exposure could potentially modify susceptibility to infection during pregnancy.

Figure 1. DCVC effects on LTA-stimulated TNF-α over time

Punches of full thickness human extraplacental membranes were floated in culture with DCVC alone or co-exposed to DCVC and LTA (1 μg/mL) for 4, 8, and 24 h, and then the medium was assayed by ELISA for TNF-α. Columns represent mean ± SEM; N=5 women. Data were analyzed by ANOVA with Tukey's post-hoc test. #, Significant differences compared to control within each time point (medium only) ($p < 0.05$). *, Significantly different compared to LTA alone within each time point ($p < 0.05$).

Figure 2. DCVC effects on LTA-stimulated TNF- α mRNA expression

Punches of full thickness human extraplacental membranes were floated in culture with and without DCVC (10 μ M) and LTA (1 μ g/mL) for 4 h, and then the RNA was extracted and assayed by PCR for TNF- α expression. Data are presented as fold change in mRNA compared to control. Columns represent mean $\pm$ SEM; N=3 women. Data were analyzed by ANOVA with Tukey's post-hoc test. #, Significantly different compared to control (medium only) ($p < 0.05$). *, Significantly different ($p < 0.05$).

Figure 3. TCA effects on LTA-stimulated release of TNF-α

Punches of full thickness human extraplacental membranes were floated in culture with TCA alone or co-exposed to TCA and LTA (1 μg/mL) for 24 h, and then the medium was assayed by ELISA for TNF-α. Columns represent mean ± SEM; N=3 women. Data were analyzed by ANOVA with Tukey's post-hoc test. #, Significant differences compared to control (medium only) (p < 0.05).

Figure 4. DCVC effects on GBS-stimulated cytokines

DCVC effects on GBS-stimulated release of pro-inflammatory cytokines from extraplacental membranes in transwell cultures. Full thickness human extraplacental membranes were cocultured with live GBS for 24 h, and then the medium from the choriodecidua compartment was assayed by ELISA for IL-1 β (A), IL-6 (B), IL-8 (C), and TNF- α (D). Columns represent mean $\pm$ SEM; N=5 women. Data were analyzed by ANOVA with Tukey's post-hoc test. #, Significant differences compared to control (medium only) ($p < 0.05$). *, Significant differences compared to GBS alone ($p < 0.05$).