BASIC SCIENCE: OBSTETRICS

Effect of *Lactobacillus rhamnosus* GR-1 supernatant and fetal sex on lipopolysaccharide-induced cytokine and prostaglandin-regulating enzymes in human placental trophoblast cells: implications for treatment of bacterial vaginosis and prevention of preterm labor

Maryam Yeganegi, HBSc; Carole S. Watson, PhD; Andrew Martins, BSc; Sung O. Kim, PhD; Gregor Reid, PhD; John R. G. Challis, PhD; Alan D. Bocking, MD

OBJECTIVE: The objective of the study was to determine the effect of fetal sex on the output of cytokines and prostaglandin-regulating enzymes in lipopolysaccharide (LPS) and probiotic lactobacilli-treated placental trophoblast cells.

STUDY DESIGN: We examined the effect of LPS and *Lactobacillus rhamnosus* GR-1 supernatant in placental trophoblast cells on tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-10 using enzyme-linked immunosorbent assay and on prostaglandin-endoperoxide synthase 2 (PTGS2), 15-hydroxy prostaglandin dehydrogenase (PGDH), and toll-like receptor-4 (TLR-4) using Western blotting. Comparisons were performed using one-way analysis of variance and Student *t* test.

RESULTS: LPS increased the output of TNF-α, IL-10, and PTGS2 with a greater response in male placentae. *L rhamnosus* GR-1 supernatant inhibited the LPS-stimulated TNF-α and increased IL-10. It also up-regulated expression of PGDH in female placentae and partially reduced the LPS-stimulated PTGS2 in male placentae. There was no change in IL-1β. Expression of TLR-4 was greater in placentae of male fetuses.

CONCLUSION: These findings suggest an underlying mechanism for the sex difference in the incidence of preterm birth and provide potential evidence for a therapeutic benefit of lactobacilli in reducing preterm labor.

Key words: cytokines, fetal sex, *Lactobacillus rhamnosus* GR-1, preterm birth, prostaglandins

Cite this article as: Yeganegi M, Watson CS, Martins A, et al. Effect of *Lactobacillus rhamnosus* GR-1 supernatant and fetal sex on lipopolysaccharide-induced cytokine and prostaglandin-regulating enzymes in human placental trophoblast cells: implications for treatment of bacterial vaginosis and prevention of preterm labor. Am J Obstet Gynecol 2009;200:532.e1-532.e8.

Preterm birth (PTB) remains a major challenge in obstetrics. It complicates up to 13% of all pregnancies[1] and accounts for approximately 80% of neonatal mortality and morbidity.[2] It has been reported that preterm birth is more frequent in women carrying a male fetus.[3,4] In addition, male fetuses are more likely to face intrauterine or neonatal death as well as other complications.[5]

PTB is multifactorial but 1 of the major causes is infection.[1,6] Bacterial Vaginosis (BV), an alteration in the endogenous vaginal microbiota associated with decreased levels of certain *Lactobacillus*,[7] is associated with a 40% increase in risk of PTB.[6] Previous studies report an increase in release of the proinflammatory cytokines associated with infection in humans, animals and in vitro models of infection-mediated PTB.[8-11] In addition, BV-associated pathogens up regulate expression of prostaglandins, leading to activation of premature uterine contraction and PTB.[12,13]

Sexual dimorphism exists in immune response to bacterial pathogens with women being more effective in mounting an immune response to pathogens and having better prognosis in septic shock than men in both neonatal and adult periods.[14-17] To date, antibiotics have been the most common treatment for BV and prevention of infection-mediated preterm birth. However, these

From the Departments of Physiology and Obstetrics and Gynecology (Ms Yeganegi and Drs Challis and Bocking), University of Toronto, Toronto; the Department of Microbiology and Immunology (Mr Martins and Drs Kim and Reid), University of Western Ontario, Siebens-Drake Research Center, and the Canadian Research and Development Centre for Probiotics, Lawson Health Research Institute, London; and the Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto (Ms Yeganegi and Drs Watson and Bocking), ON, Canada.

Presented at the 55th Annual Scientific Meeting of the Society for Gynecologic Investigation, San Diego, CA, March 28, 2008, and at the International Scientific Association for Probiotics and Prebiotics, London, UK, June 27, 2007.

Received July 11, 2008; revised Sept. 27, 2008; accepted Dec. 22, 2008.

Reprints: Alan D. Bocking, MD, Departments of Physiology and Obstetrics and Gynecology, University of Toronto, Room 1006, 600 University Ave., Toronto, ON, Canada M5G 1X5. abocking@mtsinai.on.ca.

This study was supported in part by the Canadian Institutes of Health Research Grant MOP-82799 and the Genesis Research Foundation, University of Toronto.

0002-9378/$36.00 • © 2009 Mosby, Inc. All rights reserved. • doi: 10.1016/j.ajog.2008.12.032

treatments are largely unsuccessful[18] and in some studies have been shown to increase the risk of preterm birth.[19]

A potentially novel way to protect against infection-mediated preterm birth is to use probiotic lactobacilli.[20] Probiotics defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host"[20] are being studied as a treatment alternative[20] mainly because of their ability to replenish vaginal lactobacilli and modulate immunity.[21-23]

At present, the effect of probiotics on intrauterine tissues and the mechanisms underlying the sex difference observed in the incidence of preterm birth or effectiveness of treatments are unexplored. Therefore, we sought to determine possible sexual dimorphisms in the expression of the inflammatory mediators as a result of bacterial endotoxin treatment as well as the ability of probiotic lactobacilli supernatant to antagonize these effects.

MATERIALS AND METHODS

Samples

All studies were approved by the research ethics boards of Mount Sinai Hospital and Faculty of Medicine, University of Toronto, in accordance with the Canadian Tri-Council Policy Statements on Human Ethics Reviews (institutional review board #04-0018-U). Placental tissues were collected from women undergoing term (> 37 weeks of gestation) elective cesarean section at Mount Sinai Hospital (Toronto, Canada). The subjects experienced a healthy pregnancy with no clinical infection and had no signs of labor prior to delivery. Indications for delivery by elective cesarean section included abnormal presentation of the fetus, cephalopelvic disproportion, or previous caesarean sections. Informed consent was obtained before tissue collection.

Placental trophoblast cell culture

Placental trophoblast cells were isolated using established primary culture protocols.[24] Briefly, placental tissues were separated from the membranes and washed with saline. Approximately 60 g of placental tissues were cut and digested in 37°C water bath using 0.125% trypsin (Sigma, St Louis, MO) and 0.02% deoxyribonuclease-I (Sigma) in Dulbecco's minimal essential medium (DMEM; Life Technologies, Inc, Grand Island, NY) 3 times for 30 minutes each. Supernatant was collected each time, centrifuged at 37°C, 1700 × *g* for 10 minutes, and resuspended. This was then passed through a 200 μm pore size nylon gauze filter and loaded onto a continuous Percoll (Sigma) gradient (5-70% at step increments of 5%), and centrifuged at 2500 × *g* for 20 minutes to separate different cell types. Cytotrophoblasts between the density markers of 1.049 and 1.062 g/mL were collected and washed using DMEM and centrifuged at 1700 × *g* for 10 minutes. The precipitate was then diluted using DMEM culture medium containing 10% fetal bovine serum (Sigma) and 1% antibiotic solution (Sigma).

The cells were plated in 24 well plates at a density of 10^6/well (for enzyme-linked immunosorbent assay [ELISA] measurements) or in 60 mm dishes at a density of 10^7/dish (for protein extraction). Cells were then cultured for 72 hours at 37°C in 5% CO_2 and 95% O_2. Cells aggregated to form a syncytium. The purity of the cell preparation was assessed by histochemical staining for cytokeratin, an epithelial cell lineage marker (DAKO Corp, Glostrup, Denmark), or vimentin, a mesenchymal cell lineage marker (DAKO Corp). Cells were counterstained with Carrazzi's hematoxylin. Immunohistochemical analysis showed approximately 90-95% of placental cells were cytokeratin positive as expected with a minority of vimentin stained fibroblast contamination.

Treatment of placental trophoblast cells

Supernatant from *Lactobacillus rhamnosus* GR-1 cultures was prepared as previously described.[23] Briefly, the bacteria were grown to an optical density of 1.5 at 600 nm and centrifuged for 10 minutes at 6000 × *g* at 4°C. The supernatant was then filtered through a 0.22 μm pore size filter to remove any residual bacteria. After 72 hours of trophoblast cell culture, cells were washed using DMEM media and serum starved for 12 hours. Cells were then divided into the following groups: (1) no treatment; (2) treatment with lipopolysaccharide (LPS) alone (200 ng/mL for protein or 100 ng/mL for cytokine measurements) after a further 12 hours; (3) treatment with *L rhamnosus* GR-1 supernatant alone (1:20 dilution) for 12 hours; and (4) pretreatment with *L rhamnosus* GR-1 supernatant (1:20) for 12 hours and subsequent treatment with 200 or 100 ng/mL of LPS.

Preliminary studies established dilution and time of treatments. Cell viability was assessed by Trypan Blue exclusion test and measuring lactate dehydrogenase (LDH) levels in the culture medium using commercially available LDH-cytotoxicity assay kit according to the manufacturer's instructions (BioVision, Mountain View, CA). Both methods revealed minimal cell death as a result of treatments.

Protein extraction and Western blot analysis

Eight hours following LPS treatment, media were collected from 24 well plates. Cells from 60 mm dishes were scraped off with cold radioimmunoprecipitation assay (RIPA) lysis buffer containing mini-EDTA-free protease inhibitors (Boehringer Mannheim Biochemicals, Mannheim, Germany). They were then mixed on a vortex shaker 3 times, 20 minutes apart, and centrifuged for 30 minutes at 4°C, 12,000 rpm. The supernatant was then collected. Protein concentration was measured by Bradford assay using a protein assay kit (Bio-Rad Laboratories Inc, Mississauga, ON, Canada) with bovine serum albumin as a standard. The samples were then stored at -80°C until further analysis. Twenty-five micrograms of the protein extracts were mixed with specific volumes of RIPA, and 10% loading buffer was added to bring the total volume to 20 μL.

Samples were then incubated at 55°C for 10 minutes and subjected to a standard 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories Inc). The blots were blocked overnight at 4°C with 5% nonfat