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Mesenchymal Stem Cells Suppress Inflammatory Cytokines in Lipopolysaccharide Exposed Preterm and Term Human Pregnant Myometrial Cells

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Abstract

Objective The objective of this study was to determine the cytokine response in human pregnant preterm and term myometrial cells exposed to lipopolysaccharide (LPS) and cocultured with mesenchymal stem cells (MSCs).

Study Design Myometrium was obtained at cesarean delivery in term and preterm patients. Human myometrial cells were exposed to 5 µg/mL LPS for 4 hours followed by 1 µg/mL LPS for 24 hours and were cocultured with MSCs for 24 hours. Culture supernatants were collected at 24 hours and expression of cytokines, including interleukin-1β (IL-1β), IL-6, IL-8, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and IL-10, was quantified by enzyme-linked immunosorbent assay.

Results There was significantly increased expression of the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α in preterm myometrial cells treated with LPS compared with untreated preterm myometrial cells. Coculture with MSCs significantly suppressed the proinflammatory cytokine levels in LPS-treated preterm versus treated term myometrial cells. Moreover, MSC cocultured preterm myometrial cells expressed increased levels of the anti-inflammatory cytokines TGF-β and IL-10 compared with treated term myometrial cells.

Conclusion MSCs ameliorate LPS-mediated inflammation in preterm human myometrial cells compared with term myometrial cells. Immunomodulatory effects of MSCs mediated through anti-inflammatory cytokine regulation suggest a potential cell-based therapy for preterm birth.

Keywords
- preterm birth
- inflammation
- cytokines
- mesenchymal stem cells
- immunomodulation

Human parturition is an inflammatory incident that involves production of both autocrine and paracrine factors in the gestational tissues.[1] The specific factors that initiate inflammation remain unknown but ultimately lead to a cascade of events in both term and preterm labor (PTL), including activation of membranes, uterine contractility, and cervical ripening. Inappropriate timing of inflammatory activation at the maternal–fetal interface can lead to pathological preterm birth (PTB).[2,3] This process is mediated by proinflammatory cytokines in response to various stress factors that orchestrate the activation of the parturition mechanism prematurely leading to PTL.[4,5] Myometrial inflammation plays a vital role both in

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term labor and PTL.[3,6,7] Cytokines and other mediators are considered inflammatory responses involved in cervical ripening and remodeling.[3–6]

Mesenchymal stem cells (MSCs) are multipotent cells that play a major role in modulating inflammation and immune response. Through soluble paracrine factors and therapeutic extracellular vesicles, these MSCs demonstrate potential for clinical utility.[8,9] Immunomodulatory effects of MSCs are executed by both secretory factors and direct cell-to-cell contact.[10] MSCs exert immunosuppressive effects by the production of various cytokines such as transforming growth factor-β (TGF-β), interleukin-10 (IL-10), indoleamine-2,3-dioxygenase, nitric oxide, prostaglandin2, and other soluble factors. TGF-β, IL-10 are considered potent anti-inflammatory cytokines.[11] The potential for MSCs to modulate the inflammatory and immune responses in preeclampsia has been demonstrated in both in vitro cell culture models[12–16] as well as preclinical models.[12] There have also been some studies of MSCs with recurrent pregnancy loss.[13] However, there are limited studies involving MSCs in term and preterm gestations.

Our previous work has shown that MSCs attenuates lipopolysaccharide (LPS)-mediated inflammation in human nonpregnant uterine smooth muscle cells.[17] The aim of this study was to extend our findings to pregnant uterine tissues collected at the time of term birth or PTB.

Materials and Methods

Clinical Samples

Human myometrial tissue was collected at the time of cesarean delivery from term and preterm pregnancies. This study was approved (HSC-MS14-0370) by the McGovern Medical School-UTHealth Committee for the Protection of Human Subjects. Uterine samples were collected from healthy pregnant women undergoing scheduled cesarean delivery (due to labor or preeclampsia) by transverse incision at a gestational age 37 weeks or more (term) or less than 37 weeks (preterm). Women with more than three contractions per hour, rupture of membranes, placenta previa, known infections, or uterine leiomyomas were excluded.

Cell Culture

Biopsies of $2 \times 2 \times 4$ cm were obtained from the upper edge of the lower segment of the uterine incision. Tissues were immediately placed in cold Hank's solution and transported to the lab. Cells were obtained and cultures by methods described previously.[17,18] These are reviewed briefly as follows. The uterine tissue was cut into 1- to 2-mm fragments with a razor then digested in 0.1% trypsin (Sigma, USA) and 0.1% deoxyribonuclease (Sigma, USA) for 30 minutes at 37°C in shaker incubator, followed by 0.1% collagenase (Sigma, USA) for another 30 minutes. After filtering the tissue through gauze, the cells were washed then plated on collagen I-coated T-75 mm flasks (BD Biosciences, USA) with RPMI 1640 media (Sigma, USA), 10% fetal bovine serum (Sigma, USA), and penicillin–streptomycin (Sigma, USA). The media was changed daily until Day 4.

MSCs were purchased from Promo Cell (Heidelberg, Germany) and were grown in MSC growth medium, at 37° C in a humidified atmosphere of 95% air and 5% CO_2. At 90% confluent monolayer, myometrial cells were plated at a density of 2×10^5 cells per well in a 12-well plate (Corning, NY) and treated with LPS 5 µg/mL LPS (Sigma-Aldrich, St. Louis, MO) for 4 hours, followed by 1 µg/mL LPS for 24 hours. Both preterm and term myometrial cells were divided into the following experimental groups: (1) Control (saline); (2) LPS (no MSCs); (3) MSC coculture (no LPS); and (4) LPS and MSC coculture. Following treatment with LPS or vehicle, myometrial cells were monocultured or cocultured with MSCs and plated at a total cell density of 2×10^5 cells (Corning transwell; 0.4 µm). After 24 hours, culture supernatants from myometrial cells were collected, centrifuged at 1,500 rpm for 10 minutes to remove any cell contamination and stored at −80°C until further use. Culture supernatants were assayed for expression levels of proinflammatory cytokines IL-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) and anti-inflammatory cytokines TGF-β1 and IL-10 by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) in accordance with the guidelines supplied by the manufacturer. The minimum detectable and quantifiable amount for IL-1β was 3.91 pg/mL, IL-6, 3.0 pg/mL, whereas for IL-8, IL-10, and TGF- β1 was 31.3 pg/mL, and TNF-α was 15.6 pg/mL.

Statistical Analysis

Data are expressed as mean ± standard error of the mean. Statistical analysis was carried out using Graph-Pad Prism (version 6.0, La Jolla, CA). Comparisons of cytokines expression between the above four groups were analyzed using one-way analysis of variance with Tukey's post hoc test. A p-value less than 0.05 was considered statistically significant.

Results

Myometrial cells were obtained from preterm ($n = 8$) and term ($n = 7$) subjects. Women in the preterm group delivered approximately 4 weeks earlier, were more likely to be of African America race, and had a higher rate of prior cesarean delivery (►**Table 1**). There were no differences in maternal age, gravidity, parity, or body mass index.

Inflammatory cytokines such as IL-1β were increased in preterm myometrial cells when exposed to LPS (►**Fig. 1**). LPS-exposed preterm myometrial cells expressed significantly higher levels of IL-1β compared with untreated preterm myometrial cells (LPS: 7.587 ± 0.91 vs. no LPS: 3.06 ± 0.72 pg/mL, $p < 0.001$). However, MSC suppressed inflammatory cytokines in these cells. LPS-treated preterm myometrial cells cocultured with MSCs suppressed IL-1β (LPS treated, cocultured with MSC: 5.08 ± 0.69 pg/mL vs. LPS only: 7.587 ± 0.91, $p < 0.001$; ►**Fig. 1A**).

Inflammatory cytokines such as IL-6 were increased in preterm myometrium compared with term myometrium (►**Fig. 1B**). Untreated preterm myometrial cells expressed higher levels of IL-6 than term myometrial cells (preterm: 1,267.3 ± 435.7 vs. term: 203.1 ± 90.9 pg/mL, $p < 0.001$).

Table 1 Maternal demographics

	Preterm $N = 8$	Term $N = 7$
Maternal age (y)[a]	31.4 ± 3.5	31.7 ± 2.8
Gestational age at delivery (wk)[a]	34.2 ± 2.7	38.3 ± 1.9
Gravidity[b]	4 (2–10)	3 (2–8)
Parity[b]	1 (0–1)	0 (0–1)
Race		
Caucasian[c]	12.5% (1)	14.3% (1)
African American[c]	75% (6)	28.6% (2)
Hispanic[c]	12.5% (1)	14.3% (1)
Other/multiple[c]	0% (0)	42.8% (3)
Prepregnancy BMI (kg/m^2)[a]	40.6 ± 12.9	37.1 ± 11.6
Prior cesarean delivery[a]	87.5% (7)	71.4% (5)

Abbreviation: BMI, body mass index.
[a]Mean ± standard deviation.
[b]Median (range).
[c]Percent (*N*).

LPS-exposed preterm myometrial cells cocultured with MSCs significantly suppressed IL-6 expression (LPS treated, exposed with MSC: 525.2 ± 78.3 pg/mL vs. LPS only: 3,335.8 ± 280.4, $p < 0.001$). Expression levels of IL-8 increased significantly in preterm myometrial cells exposed to LPS and were attenuated in MSC cocultures (LPS treated, cocultured with MSC: 1,417.9 ± 40.5 pg/mL vs. LPS only: 2,756.0 ± 199.9, $p < 0.001$, ►**Fig. 1C**). Similar findings were seen with TNF-α (LPS treated, cocultured with MSC: 9.1 ± 1.1 pg/mL vs. LPS only: 498.4 ± 34.0, $p < 0.001$, ►**Fig. 1D**).

Preterm myometrial cells cocultured with MSCs expressed significantly increased anti-inflammatory cytokines TGF-β1 (LPS treated, cocultured with MSC: 884.7 ± 179.4 pg/mL vs. LPS only: 40.8 ± 3.5, $p < 0.001$, ►**Fig. 2A**). Similar findings were seen with IL-10 expression (LPS treated, cocultured with MSC: 159.87 ± 7.1 pg/mL vs. LPS only: 23.30 ± 2.4, $p < 0.001$, ►**Fig. 2B**).

Discussion

The results of our in vitro study demonstrate that human pregnant myometrial cells treated with LPS and cocultured with MSCs express significantly reduced levels of the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α and significantly increased levels of the anti-inflammatory cytokines TGF-β and IL-10. The effects were enhanced in preterm myometrial cells compared with term myometrial cells. Moreover, these findings align with our prior work demonstrating attenuation of inflammatory cytokines and elevation of anti-inflammatory cytokines in LPS-exposed and treatment with MSCs in human nonpregnant myometrial cells.[17]

The unique characteristic feature of MSCs are self-renewal and multilineage differentiation that together create an enduring therapeutic immunomodulatory role. The

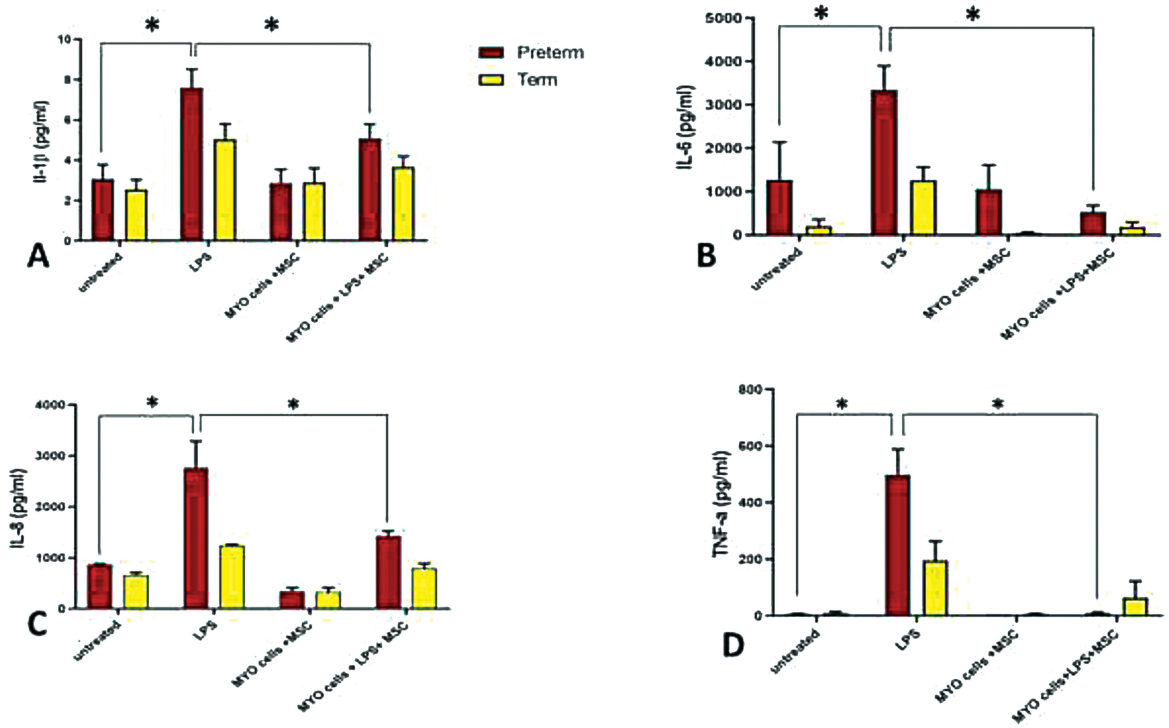

Fig. 1 The effect of mesenchymal stem cells (MSC) on proinflammatory cytokines in preterm human myometrial cells (MYO) treated with lipopolysaccharide (LPS). Preterm myometrial cells showed significant decrease in proinflammatory cytokine levels of IL-1β (**A**), IL-6 (**B**), IL-8 (**C**), and TNF-α (**D**) measured using enzyme-linked immunosorbent assay. Data represent mean ± standard error of mean. *p-Value < 0.001.

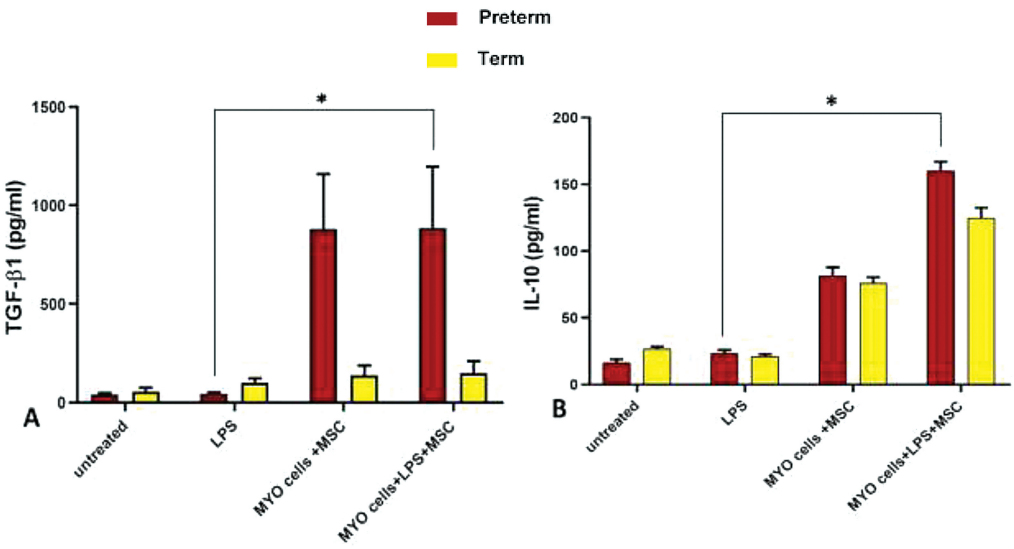

Fig. 2 Effect of mesenchymal stem cells (MSC) on preterm and term human myometrial cells (MYO) treated with lipopolysaccharide (LPS). Preterm myometrial cells showed an increase in anti-inflammatory cytokine levels transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) (**A**–**B**). Data represent mean ± standard error of mean. *p-Values < 0.001.

immunosuppressive properties of MSCs as potential novel modulators of PTB leverage their prime role in innate immunity.[17] Innovative tissue engineering technologies have led to the production of MSC 3D scaffolds that promote secretion of anti-inflammatory cytokines and reduce inflammatory cell infiltration in nonobstetric applications.[19] MSCs have been shown to exert therapeutic effects in diseases of various organs, including the heart, lung, and liver. Growing evidence has shifted toward paracrine factors and extracellular vesicles being responsible for mediating immunomodulatory and regenerative MSC functions. Novel technologies allow the large-scale production of MSCs in bioreactors. MSC can also be applied, with or without scaffolds, in tissue engineering concepts for disease modelling and therapy.[20]

This study used an in vitro LPS experimental model to investigate the therapeutic potential of MSCs for PTB. Bacterial infections and pathological inflammation are some of the leading factors leading PTB[21–23] and novel cell-based therapies for high-risk women with inflammation-mediated PTB may well be benefited over the current therapeutics. Our observation that MSC's suppress proinflammatory cytokines is consistent with previous findings in both in vitro[24–26] and in vivo studies[5,27,28] in various inflammatory conditions. Prior studies suggest that MSC's modulatory effects of immune response is mediated by soluble factors.[9] The current study showed that TGF-β1 and IL-10 levels were significantly increased in LPS-treated preterm myometrial cell treatments. This suggests that IL-10 may be a major anti-inflammatory cytokine contributor to the establishment and maintenance of immunosuppression necessary for endometrial receptivity.[29] Evidence supports that IL-10 delays the onset of preterm, but the driving mechanism remain elusive.[30] There are a few contradicting reports on the role of TGF-β and IL-10 in PTB. Some studies show low TGF-β levels, and high IL-10 were found in blood from the umbilical cord of pregnant women with placental inflammation, whereas other studies have shown low IL-10 levels in blood from the umbilical cord of preterm neonates.[31] Moreover, others have found high IL-10 in the plasma of pregnant women who experienced PTB, and higher levels of TGF-β were associated with increased odds of PTB at less than 35 weeks' gestation.[31,32]

There are some strengths and weaknesses to this study. A strength is that we were able to test the effects of MSCs on the cytokine effect from actual pregnant myometrial tissue, both preterm and term, for this in vitro study. One weakness is that is it not clear whether the causes for these pregnant women who delivered prematurely were due to spontaneous labor or medically indicated deliveries (such preeclampsia, fetal growth restriction, or abnormal fetal antenatal testing). Thus, we cannot assume that our results apply to just spontaneous PTL. Another weakness is that the myometrial tissue was collected only from pregnant women who underwent a cesarean delivery. We cannot assume that results would be similar in pregnant women who deliver via vaginal delivery.

MSC's exert immunomodulatory effects mediated by the production of IL-10, TGF-β, and several other soluble factors primarily by their capacity of adapting and regulating in a manner specific to the cellular environment in which they localize. MSCs may function as therapeutics with clinical benefits in PTB, intrauterine infection, premature rupture of membrane, and other inflammation-related reproductive complications. Our results suggest that MSCs may be potential novel cellular therapeutics. Further in vitro and in vivo studies are required for the translation of these basic scientific findings into therapeutic interventions. A logical next step may be to eliminate the need for MSC coculture by directly treating myometrial cells with MSC-derived extracellular vesicles purified from culture supernatants; this approach has enhanced therapeutic potential as MSC-derived Extracellular Vesicles (ECVs) can be administered intravenously.[33,34]

Conflict of Interest
None declared.

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