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Uterine overdistention induces preterm labor mediated by inflammation: observations in pregnant women and nonhuman primates

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Abstract

OBJECTIVE—Uterine overdistention is thought to induce preterm labor in women with twin and multiple pregnancies, but the pathophysiology remains unclear. We investigated for the first time the pathogenesis of preterm birth associated with rapid uterine distention in a pregnant nonhuman primate model.

STUDY DESIGN—A nonhuman primate model of uterine overdistention was created using preterm chronically catheterized pregnant pigtail macaques (*Macaca nemestrina*) by inflation of intraamniotic balloons (N = 6), which were compared to saline controls (N = 5). Cesarean delivery was performed due to preterm labor or at experimental end. Microarray, quantitative reverse

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transcriptase polymerase chain reaction, Luminex (Austin, TX), and enzyme-linked immunosorbent assay were used to measure messenger RNA (mRNA) and/or protein levels from monkey (amniotic fluid, myometrium, maternal plasma) and human (amniocytes, amnion, myometrium) tissues. Statistical analysis employed analysis of covariance and Wilcoxon rank sum. Biomechanical forces were calculated using the law of Laplace.

RESULTS—Preterm labor occurred in 3 of 6 animals after balloon inflation and correlated with greater balloon volume and uterine wall stress. Significant elevations of inflammatory cytokines and prostaglandins occurred following uterine overdistention in an “inflammatory pulse” that correlated with preterm labor (interleukin [IL]-1 β , tumor necrosis factor [TNF]- α , IL-6, IL-8, CCL2, prostaglandin E2, prostaglandin F2 α , all $P < .05$). A similar inflammatory response was observed in amniocytes in vitro following mechanical stretch (IL1 β , IL6, and IL8 mRNA multiple time points, $P < .05$), in amnion of women with polyhydramnios (IL6 and TNF mRNA, $P < .05$) and in amnion (TNF- α) and myometrium of women with twins in early labor (IL6, IL8, CCL2, all $P < .05$). Genes differentially expressed in the nonhuman primate after balloon inflation and in women with polyhydramnios and twins are involved in tissue remodeling and muscle growth.

CONCLUSION—Uterine overdistention by inflation of an intraamniotic balloon is associated with an inflammatory pulse that precedes and correlates with preterm labor. Our results indicate that inflammation is an early event after a mechanical stress on the uterus and leads to preterm labor when the stress is sufficiently great. Further, we find evidence of uterine tissue remodeling and muscle growth as a common, perhaps compensatory, response to uterine distension.

Keywords

amniocyte; amnion; chemokine (C-C motif) ligand 2; choriodecidua; cytokines; interleukin-1; interleukin-8; *Macaca nemestrina*; monocyte chemotactic protein 1; myometrium; pregnancy; preterm labor; prostaglandin E2; prostaglandin F2 α ; tumor necrosis factor; uterine stress; uterine stretch

Preterm delivery represents a significant public health and economic burden to our society. Preterm birth (PTB) occurs in 11.4% of births and is associated with neonatal mortality and long-term morbidity.¹ Over the course of gestation, uterine myocytes normally undergo hypertrophy and hyperplasia to accommodate the relatively slow-growing fetus, placenta, and amniotic fluid.^{2,3} However, women tend to deliver preterm if they carry twins (54% rate),⁴ have polyhydramnios, or have uterine anomalies, such as a unicornuate uterus.⁵⁻⁷ Hence, there appears to be a limit to the degree the uterus can increase in size, and we propose that one of the factors predisposing women to PTB is an inability of the uterus to adapt to a continued increase in volume. A lack of appropriate animal models and the complex nature of hormonal and inflammatory factors coordinating the onset of labor have made it difficult to investigate the pathogenesis of preterm labor associated with increased uterine volume, sometimes referred to as excessive stretch. Although the idea of uterine stretch is intuitive for many obstetricians to describe the force of increasing uterine volume on the myometrium, the correct biomechanical term is “stress” (force per unit area).⁸ Therefore, we have avoided the term uterine “stretch” and instead refer to myometrial stress, over-distention, or increasing uterine volume.

The use of extraamniotic balloons in human beings to increase uterine wall stress is well known to trigger abortion, labor at term, or uterine contractions postpartum. Extraamniotic balloon inflation induces abortion in the late second trimester with nearly half of subjects aborting within 24 hours.⁹ At term, labor is often rapidly initiated in healthy women with extraamniotic balloon inflation and the volume of inflation correlates with a more rapid delivery.¹⁰ Postpartum, balloon inflation also induces regular uterine activity, which was suppressed by indomethacin, supporting a central role for prostaglandins in this pathway of myometrial activity.¹¹ Ethical considerations in human studies prevent serial sampling of myometrium or amniotic fluid in the same patient; therefore, human studies are generally cross-sectional in design. This limits the ability to establish temporal relationships among increases in uterine volume, production of inflammatory mediators, and the evolution of preterm labor. As a result, mechanisms of preterm labor in women with polyhydramnios and multiple gestation are understudied, despite the fact that multiple gestations have a significant risk of PTB.¹²

Both rat and marsupial models of increasing uterine volume have been described, providing a greater opportunity to discover the underlying mechanisms involved. In the rat model, animals first undergo unilateral tubal ligation to ensure that they only become pregnant in a single uterine horn.¹³ After becoming pregnant, a second procedure places a tube into the nongravid uterine horn to simulate increasing uterine volume. In the tammar wallaby, only a single uterine horn becomes pregnant naturally and one may compare samples between the nongravid horn with the gravid horn undergoing gradual expansion of the uterine volume during the pregnancy.¹⁴ These studies and experiments in cultured human myometrial cells identified important roles for CCL2 (chemokine [C-C motif] ligand 2, also known as monocyte chemoattractant protein 1), oxytocin receptor, and connexin-43.¹⁴⁻¹⁷ However, biological differences in parturition between lower mammalian models and human beings/nonhuman primates present difficulty in translating these results to human pregnancy. For example, in women and primates, progesterone levels do not decline in maternal or fetal blood just prior to labor onset, as they do in almost all other animal species.¹⁸⁻²⁰ Progesterone modulates the expression of many genes associated with labor onset making it difficult to translate results of other animal studies to human parturition. These limitations have been overcome in our nonhuman primate model, where we have previously described infection-induced hormonal and immunologic events culminating in PTB.²¹

Our objective was to identify early biological events occurring in the amniotic fluid and myometrium after an increase in uterine volume induced by inflation of an intraamniotic balloon in a nonhuman primate (Video). We hypothesized that inflation of an intraamniotic balloon would increase uterine wall stress and trigger preterm labor with a unique pattern of mediators in the amniotic fluid and myometrium. A unique gene activation pattern due to stretch in uterine smooth muscle has yet to be elucidated, but such patterns have been described in vitro using primary cultures or muscle strips from cardiomyocytes, bladder, and airway smooth muscle.²²⁻²⁴ Further, we used human amniocytes that were stretched in vitro and amnion and myometrial samples from women with twins or polyhydramnios to validate and extend our results.

Materials and Methods

Animals and study groups

Eleven chronically catheterized pregnant pigtail macaques (*Macaca nemestrina*) at 118-125 days' gestation (term = 172 days) received 1 of 2 experimental treatments: choriodecidual and intraamniotic saline infusions (n = 5), or intraamniotic balloon inflation with saline (n = 6; varying protocols) (Table 1). In our model, pregnant pigtail macaques were time-mated and fetal age determined using early ultrasound. The tethered chronic catheter preparation was used for all in vivo experiments and is a major breakthrough in studying maternal-fetal immunologic responses.^{21,25} Details of our model have been described elsewhere²⁶; we describe slight modifications necessary to create the balloon inflation model. The animal was first conditioned to a nylon jacket/tether system for several weeks before surgery. On day 118-125 of pregnancy (term = 172 days) a maternal temperature probe and catheters were implanted into the maternal femoral vein and amniotic cavity. Two or 3 intraamniotic balloons were implanted into the amniotic fluid per protocol (Table 1). Care was taken to make the smallest uterine incision possible to accommodate these catheters, usually 3-4 cm in length. The saline controls did not have the intraamniotic balloons inserted, but instead had amniotic fluid, choriodecidual, maternal femoral vein and fetal vein catheters, and 1 temperature probe implanted. After surgery, the catheters were tracked through the tether system and cefazolin and terbutaline sulfate were administered to reduce postoperative infection risk and uterine activity. Terbutaline was stopped at least 72 hours before experimental start (~5 half-lives for terbutaline, 40 half-lives for cefazolin, >97% of both drugs eliminated). Cefazolin (1 g) was administered intravenously each day to minimize chances of a catheter-related infection. Experiments began approximately 10 days to 2 weeks after catheterization surgery to allow recovery. At our center, term gestation in the noninstrumented pigtail macaque population averages 172 days. Catheterization surgery (118-125 days) and experimental start (128-135 days) are roughly equivalent to 28 and 30 weeks' human gestation, respectively. The saline control group experiments were performed a few years before the uterine overdistention experiments and were not repeated to allow for randomization due to ethical and financial constraints. The saline control animals originally received 2 inoculations (amniotic fluid, choriodecidual) to demonstrate that neither inoculation was associated with elevated cytokines or prostaglandins.²⁶

Intraamniotic balloon

A 22-French Kenguard silicone-coated Foley catheter (Kendall Co, Mansfield, MA) designed for the human bladder was modified to create an intraamniotic balloon. First, the Foley catheter was cut close to the balloon and then a polyvinyl 1-mm catheter was glued into the Foley catheter channel to allow for balloon inflation. We determined that this Foley balloon could be inflated with 80 mL of saline and despite a variety of mechanical stresses remain inflated for >6 weeks on the bench (data not shown).

Uterine activity, labor and delivery

Intraamniotic pressure, fetal heart rate, and maternal temperature were continuously recorded and digitized with a Powerlab System (AD Instruments, Colorado Springs, CO) connected to a computer. Amniotic fluid pressure signals were processed after delivery

using custom software to eliminate noise due to respiration or position changes. The area under the pressure – time curve (mm Hg × seconds, contraction area) was calculated to directly measure the strength of each contraction. To account for changes in the frequency of contractions, and to help determine how total uterine activity changes over time, we calculated the average mm Hg × seconds generated over each hour. This method of quantifying uterine activity yields numeric values with units of mm Hg × s/h, and reflects the average of the total uterine activity occurring in each hour. Labor was defined as progressive uterine activity associated with cervical effacement and dilatation (at least 1 cm dilated). For reference, the preterm macaque fetus (~200-300 g) will deliver once the cervix is approximately 4 cm dilated. We further defined labor as associated with balloon inflation if labor occurred within 10 days of initial balloon inflation. Cervical examinations were performed on the day prior to inoculation and if labor was suspected based on uterine activity. Cesarean delivery was performed in all animals to optimize the collection of intact gestational tissues except 1 uterine stretch case that delivered spontaneously overnight. In saline controls, the cesarean delivery was performed 7 days after initial inoculation; saline was inoculated twice (day 0 and day 4) in alternating order into the choriodecidual space and amniotic fluid. At the time of cesarean delivery, the intraamniotic balloons were inspected and it was noted whether or not they were inflated. The amount of amniotic fluid at the time of catheterization surgery and cesarean delivery was recorded. Amniotic fluid was collected frequently over the course of the experiment as previously described.²⁶ Fetal blood and tissues (lungs, meninges) were also cultured at the time of cesarean delivery. After cesarean delivery, fetuses were euthanized by barbiturate overdose followed by exsanguination and fetal necropsy. Complete gross and histopathologic examination was performed on infants and placentas. A board-certified veterinary pathologist (A.E.B.) examined the placenta and uterine tissues.

Quantitation of amniotic fluid cytokines and prostaglandins

Amniotic fluid and maternal and fetal blood were sampled frequently before (–24 and –1 hour) and after (+1, +6, +12, +24 hours, and then daily until delivery) balloon inflation. The amniotic fluid catheter malfunctioned in 1 animal preventing amniotic fluid sampling. Amniotic fluid and blood samples were centrifuged for 5 minutes at 1200 rpm and the supernatant frozen and stored at –80°C. Prior to freezing, ethylene diamine tetra-acetic acid (8.7 mmol/L) and indomethacin (0.3 mmol/L) was added to samples saved for cytokine and prostaglandin quantitation, respectively. Cytokine (interleukin [IL]-1 β , IL-6, IL-8, and tumor necrosis factor [TNF]- α) levels were determined by Luminex multiplex technology (Austin, TX) using commercially available nonhuman primate cytokine kits (Millipore, Billerica, MA). Quantities of prostaglandin E2 (PGE₂) and prostaglandin F2 α (PGF_{2 α}) were determined using commercially available human enzyme-linked immunosorbent assay kits (Cayman Chemical, Ann Arbor, MI). CCL2 levels were determined using the commercially available human enzyme-linked immunosorbent assay kit (R&D Systems Ltd, Minneapolis, MN). Values were converted to pg/mL by comparison to a standard curve.

Biomechanical modeling

The purpose of performing biomechanical calculations is to quantify the relative amounts of added tissue stress induced by the different protocols of the experiments. This is important,

since the ability of the myometrium to adapt is likely dependent on the relationship between the volume change and the mechanical reserve of the tissue. Additionally, the baseline intrauterine pressure may be critically important in the initiation of labor.^{27,28}

The physiological loads on the uterus following inflation of a balloon can be divided into 3 stages. First, balloon inflation increases the intrauterine volume, which abruptly increases intrauterine pressure and tension on the wall of the uterus. From a technical perspective, the added volume increases stress on the uterine wall according to the stress-strain relationship of the tissue. Stress is the tangential force per unit area. The second stage occurs over the course of several minutes, when the tissue lengthens to accommodate the new volume through a process known as visco-elastic creep. During this stage, the intrauterine pressure and tissue stress both fall from the peak values occurring immediately after balloon inflation, but remain higher than prior to balloon inflation. Finally, over hours or days, a third stage may occur, where the myometrium adapts to the conditions by tissue remodeling. Eventually it may achieve intrauterine pressure and wall stress that are similar to the conditions before balloon inflation.

The law of Laplace can approximate the relationship between intrauterine pressure and wall stress throughout these stages. Data values for creep, strain, and stress for myometrium of pregnancy are not available for the macaque, so we approximated these values from the best available human data.^{28,29} Resting intrauterine pressures have previously been measured by amniocentesis in human gestation.³⁰ Between 30-34 weeks, pressures measured at the abdominal surface averaged 6 torr. The mean intrauterine pressure is higher than at the abdominal surface, because there is a loss of pressure as the fluid rises (gains elevation). The distance from the abdominal surface to the center of the uterine cavity is ~8-10 cm, so it is necessary to add the pressure corresponding to this lower height to obtain the mean intrauterine pressure in the center of the cavity. Thus, adding the pressure of 8 cm H₂O (6 torr) to the 6 torr measured at the abdominal surface yields a typical resting intrauterine pressure of approximately 12 torr (1600 Pa). We used this value as the resting intrauterine pressure before balloon inflation in our calculations for each experiment.

To approximate the conditions through the first 2 stages following balloon inflation, the following calculations were performed:

- Stage 1, balloon inflation. Before inflation, intrauterine pressure was approximated to be 12 torr. Uterine wall stress was calculated from the volume using the law of Laplace for a sphere: $T = P \cdot r / 2w$, where T is wall stress, P is intrauterine pressure, r is the radius of the sphere, and w is wall thickness. For the relatively small changes of r encountered in this work, changes of w were small and were ignored. After balloon inflation, the new surface area of the uterus was calculated from the new volume. The new stress was calculated from the stress-strain relationship reported for the internal os (opening) of the pregnant human cervix.²⁹ A post-inflation intrauterine pressure was then recalculated using the law of Laplace.
- Stage 2, tissue creep. Isolated myocytes can creep to 55% of the stress values for peak stress.³¹ Since the dominant component of the wall of the uterus is the

myocyte, we will use this value to approximate tissue creep. The intrauterine pressure was then recalculated using the postcreep value for stress.

An additional assumption must be made to address the anatomy of the macaque uterus. The calculations were performed using the same wall thickness (3 mm) throughout the uterus, although our measurements demonstrate that wall thickness varied between 2.9-4.2 mm in the fundal area, and is approximately 2 mm in the lower uterine segment. Balloon inflation preferentially increases wall stress over the thinner parts of the wall, and partly spares the thicker regions. However, because we are unsure of the anatomic details regarding the distribution of the wall thinning in each animal, we are unable to assess the distributions of the peak stresses. Because of this assumption, we suggest that calculated pressures be interpreted as an upper limit, and the stress increases be interpreted as a lower limit. We further refined our estimates of uterine wall stress using the weight of the fetus and amniotic fluid volume in each experiment. For unavailable data, average values of the other experiments were used. We estimated the placental volume for the preterm macaque to be 75 mL.

RNA extraction and microarray processing

To study genetic pathways in *Macaca nemestrina*, we used the rhesus macaque array (GeneChip rhesus macaque genome array, Affymetrix, Santa Clara, CA), which allows interrogation of 47,000 *Macaca mulatta* transcripts and provides comprehensive transcriptome coverage. Genetic differences between *Macaca mulatta* and *Macaca nemestrina* are predicted to be <1%, which is consistent with our published data.³² RNA extraction was performed using miRNeasy mini kits (Qiagen, Valencia, CA) following the manufacturer's established protocol for purification of total RNA from animal tissues. RNA integrity was assessed with a 2100 Bioanalyzer (Agilent, Santa Clara, CA) and quantified with Nano-Drop 8000 (Thermo Scientific, Waltham, MA). Processing of RNA samples was carried out according to the Affymetrix GeneChip 3'IVT express kit protocol. Briefly, these methods include the synthesis of first- and second-strand complementary DNAs (cDNAs), the purification of double-stranded cDNA, the synthesis of complementary RNA (cRNA) by in vitro transcription, the recovery and quantitation of biotin-labeled cRNA, the fragmentation of this cRNA and subsequent hybridization to the micro-array slide, the posthybridization washings, and the detection of the hybridized cRNAs using a streptavidin-coupled fluorescent dye. Hybridized Affymetrix arrays were scanned with an Affymetrix GeneChip 3000 fluorescent scanner. Image generation and feature extraction was performed using Affymetrix GeneChip Command Console software.

Single gene analysis

The data discussed in this publication have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/index.cgi>) and are accessible through GEO series accession number GSE63274.³³ Analysis of the microarray data focused first on differential expression of single genes. Raw microarray data were preprocessed and analyzed with the Bioconductor (<http://www.bioconductor.org/>)³⁴ oligo package.³⁵ Several quality control steps were carried out to insure that the data were of high quality: (1) visual inspection of the

chip pseudoimages generated by the Bioconductor affyPLM package³⁶; (2) generation and inspection of histograms of raw signal intensities; (3) principal components analysis plots of the normalized data; and (4) generation and comparison of the relative log expression and normalized unscaled SE using the Bioconductor affyPLM package.^{36,37} The data were normalized and summarized using a robust multiarray average.³⁸ From the normalized data, genes with significant evidence for differential expression were identified by fitting a weighted analysis of variance (ANOVA) model using the Bioconductor limma package.³⁹ We first fit linear model (specifically an ANOVA) to the gene expression data and by incorporating weights we could smoothly up- or down- weight different samples based on similarities between the sample and others of the same type.⁴⁰ *P* values were calculated with a modified *t* test in conjunction with an empirical Bayes method to moderate the SE of the estimated log-fold changes. Genes were considered significantly differentially expressed at an unadjusted *P* value < .05 and a 2-fold difference.

Validation of cDNA microarray by quantitative reverse transcriptase polymerase chain reaction

Quantitation of messenger RNA (mRNA) levels was performed by the Center on Human Development and Disability Genomics Core Laboratory (University of Washington, Seattle, WA) using fluorogenic 5' nuclease-based assays and has been previously described.⁴¹⁻⁴³

Self-contained gene set test

A self-contained gene set test was performed on 3 gene sets that were hypothesized to be affected by uterine distension (2 terms from the Gene Ontology Biological Process, "angiogenesis" and "muscle development," as well as the Reactome myogenesis pathway) using the mroast function⁴⁴ from the Bioconductor limma package. A self-contained gene set is based on the null hypothesis that no genes are differentially expressed against the alternative hypothesis that 1 genes are differentially expressed. To assess significance, the null distribution is estimated via random rotations of the orthogonalized residuals from the ANOVA model.

Ingenuity Pathway Analysis

We used the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA) to discover pathways and transcriptional networks in the gene expression microarray data. Our data set containing gene identifiers and corresponding expression changes between the experimental groups and *P* values was uploaded into the IPA application. Each identifier was mapped to its corresponding object in the Ingenuity knowledge base. The functional analysis identified the biological functions and/or diseases that were most significant to the data set. Genes from the data set with >1.5-fold differential expression (up-/down-regulation) and *P* < .05 that were associated with biological functions and/or diseases in the Ingenuity knowledge base were considered for the analysis. The categories "top molecular and cellular functions," "physiological systems," "top canonical pathways," and "top transcription factors" were primarily used in this analysis. Right-tailed Fisher exact test was used to calculate a *P* value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. IPA also

allows prediction of the activation or inhibition of transcription factors involved in the gene expression patterns seen in our study.

Human tissues

For experiments involving amnion epithelial cell cultures, the methods have previously been described for procurement of intact human fetal membranes from term elective cesarean deliveries before labor and cell culture.⁴⁵ Preterm human amnion and myometrial tissues from women with (N = 4) and without (N = 36) polyhydramnios delivered by cesarean delivery were obtained. Polyhydramnios was defined as excessive amniotic fluid as measured by an amniotic fluid index >24 cm. In the group with polyhydramnios, the women were in early preterm labor (maximum cervical dilation of 3 cm), but the fourth was not in labor. Control tissues were obtained from women without polyhydramnios, who delivered preterm before the onset of labor (N = 36) for other indications (preeclampsia, intrauterine growth restriction). The mean gestational age of the women with polyhydramnios and preterm controls was similar (32.3 ± 1.4 vs 33.1 ± 2.7 weeks).

The human amnion and myometrial samples from women with twins were obtained at the time of cesarean delivery from 15 women not in labor and 10 women in early preterm labor. The myometrial tissues were collected from the upper aspect of the lower uterine segment immediately after hysterotomy. Preterm labor was defined as the presence of regular uterine contractions with evidence of cervical dilation, which ranged from 1-3 cm. The mean gestational age of the women with twins in preterm labor and not in labor were similar (34.3 ± 1.6 vs 35.4 ± 1.2 weeks).

RNA extraction, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays have been previously published.⁴⁵ Primer sequences for angiotensin II receptor type 1 (*AGTRI*), ephrin type-B receptor 1 (*EPHBI*), heparin-binding epidermal growth factor-like growth factor (*HBEGF*), membrane metalloendopeptidase (*MME*), and transforming growth factor, beta-induced (*TGFBI*) are shown in Supplemental Table. Cytokine and chemokine levels in the myometrial and amnion tissues were determined by Luminex multiplex technology using commercially available human cytokine/chemokine kits (Biorad, Hercules CA). The number of cases and controls used in the polyhydramnios or twins pregnancy protein and mRNA analyses varied slightly from the total cohort due to availability of tissues for both analyses. Final numbers for each group are noted in the figure legends.

In vitro stretch of human amniocytes

Static stretch was applied to the amnion cell monolayers using a Flexercell strain unit (FX-4000; Flexcell International Corp, McKeesport, PA). The stretch unit consists of a computer-controlled vacuum unit and a BioFlex base plate (to hold the 6-well culture dishes; Flexcell International Corp., Burlington, NC) and gaskets, BioFlex loading stations, and BioFlex plates. The base plates are placed in a humidified incubator with 5% carbon dioxide at 37°C. The system applies strain using a vacuum to deform the flexible membrane on which cells are cultured.

The BioFlex culture plate used was a 6-well culture plate with an optically clear, thin membrane (0.02-in thick) of rubber coated with collagen type I. The BioFlex loading stations are designed to provide uniform radial and circumferential strain across the membrane surface up to 20% elongation. They are composed of a single plate and 6 individual, removable planar cylinders (6 loading posts) per plate. The membrane is pulled over the post by the vacuum; each radius is stretched by same amount at any point in the circle thus providing uniform “equibiaxial” strain for all cells on the membrane.

Confluent human amnion epithelial cells were plated on flexible type I collagen coated plates and were subjected to static stretch and 11% elongation. An 11% stretch was the maximum amount of stretch that could be used without the amnion cells being lifted off the culture plates and dying. Non-stretched cells were used as controls and cultured under identical conditions but were not subjected to mechanical stimulation. Time periods of up to 6 hours of stretch were used for the studies. After 6 hours of stretch, >90% of cells were viable as measured by trypan blue exclusion and >99% of cells remained attached to the 6-well culture plates after stretch protocols.

RNA extraction, qRT-PCR assays, and primer sequences for IL-1 β , IL-8, and glyceraldehyde 3-phosphate dehydrogenase have been previously published.⁴⁵ Primer sequences for TNF and IL-6 are shown in Supplemental Table. Note that IL-8 mRNA levels due to stretch have been previously reported by our group,⁴⁵ but our work here represents independent experiments not previously published.

Statistical analysis

Study outcomes were quantities of uterine activity, cytokines/chemokines, and prostaglandins in amniotic fluid (IL-1 β , TNF- α , IL-6, IL-8, CCL2, PGE₂, PGF_{2 α}). For uterine activity, we specifically compared mean 24-hour hourly contraction area in the first 10 days after balloon inflation to isolate the effect of balloon-induced uterine strain from confounding effects associated with advancing gestation. We used analysis of covariance to assess differences between postinoculation peak values for control and balloon groups with adjustment for each animal's baseline value immediately prior to balloon inflation. *P*-values were based on analysis of covariance models adjusting for baseline fit on log-transformed values. A Wilcoxon rank sum test was used for the mRNA qRTPCR related to the microarray analyses. All of these statistical analyses were conducted using Intercooled STATA 8.2 for Windows 2000 (StatCorp, College Station, TX). Ratios for mRNA or protein levels to glyceraldehyde 3-phosphate dehydrogenase between unstretched and stretched cells were assessed by Wilcoxon signed ranks test (nonparametric test for related samples) using SPSS (Version 10.0; IBM Corp, Armonk, NY). Significance was considered at *P* < .05. Adjustment for multiple comparisons was not performed given the limited sample size.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council and the Weatherall Report, “The Use of Nonhuman Primates in Research.” The protocol was

approved by the University of Washington Institutional Animal Care and Use Committee (permit no. 4165-01). All surgery was performed under general anesthesia and all efforts were made to minimize suffering. Human fetal membranes and myometrial samples were obtained from women with approval from the local ethics committee (Regional Research Ethics Committee 6283, London-Chelsea Ethics Committee 10/H0801/45, approved Dec. 23, 2010). Informed consent was obtained from patients before the collection of tissues.

Results

Uterine activity, cytokines, and prostaglandins

As this was the first study of uterine overdistention in a nonhuman primate, balloon inflation protocols varied as we gathered more information about the effects of uterine distention on preterm labor. Initially, a slow inflation protocol was tried, but when labor did not occur after 18 days, a more rapid inflation protocol was used for the remaining experiments. Experimental results correlating balloon volume, labor, and days until delivery are shown in Table 1. The mean gestational age at the time of saline inoculation or balloon inflation was similar (saline 135 days, balloon 133 days) corresponding to approximately 80% of a typical *Macaca nemestrina* gestation in our colony (term ~172 days). The uterus was quiescent in all 11 animals prior to balloon inflation or saline inoculation. Within 10 days of balloon inflation, preterm labor occurred in 3 of 4 animals experiencing the greatest uterine distention due to inflation of 3 balloons. There was a notable outlier of a single animal delivering at term despite inflation of 3 intraamniotic balloons (animal no. 3) (Table 1). Intrapartum fetal demise occurred in 2 of 3 animals after inflation of 3 balloons. In 1 case, this was due to an obvious cord entanglement with the intraamniotic catheters (animal no. 4) (Table 1). In the second case, the cause was unknown, but presumably was due to cord compression by the inflated balloon (animal no. 5) (Table 1). Our results suggest that greater uterine distention is associated with preterm labor, but progression to term in some cases may occur with a similar degree of uterine stress.

Temporal relationships among uterine activity, amniotic fluid cytokines, and prostaglandins are depicted in 1 animal after saline inoculation and 2 following balloon inflation (Figure 1). Saline infusion in either the choriodecidual space or the amniotic fluid of controls was not associated with an elevation in amniotic fluid cytokines (Figure 1A). Rapid balloon inflation was associated with significant elevations of IL-1 β , TNF- α , IL-8, CCL2, PGE₂, and PGF_{2 α} in the amniotic fluid following balloon inflation and preceding labor (Figure 1B, and Table 2) (IL-1 β , P .003; TNF- α , P = .02; IL-6, P = .02; IL-8, P = .001; CCL2, P = .007; PGE₂, P = .001; PGF_{2 α} , P = < .001). The temporal profile of amniotic fluid cytokine and prostaglandin elevations more closely resembled an “inflammatory pulse” than a sustained and building inflammatory event typical of infection-associated PTB in nonhuman primates.²¹ Levels of PGE₂ and PGF_{2 α} in the amniotic fluid remained slightly higher following the pulse than at baseline.

The peak levels of cytokines and prostaglandins correlated with the volume of uterine distention. Small volumes of balloon inflation induced a small rise or no change in cytokines and prostaglandins and were not associated with labor. There were no significant differences in amniotic fluid cytokines or prostaglandins between the balloon group not in labor by 10

days and controls. In 1 animal undergoing slow distention, a rise in IL-8 was only evident after greater volumes were added to the balloon in the final 2 days before cesarean delivery (Figure 1C). These results illustrate that preterm labor after balloon inflation is associated with higher levels of amniotic fluid proinflammatory cytokines, chemokines, and prostaglandins. The pattern of a rise in amniotic fluid cytokines, chemokines, and prostaglandins following a uterine stress induced by balloon inflation and preceding labor suggests that inflammation may be a trigger of parturition for this type of uterine stress. The observation that smaller volumes of balloon inflation can also induce a rise in cytokines also suggests that there is an inflammatory threshold, which must be reached to trigger labor.

Fetal plasma cytokines were available at the time of cesarean delivery in 2 cases after balloon inflation (animals no. 2 and 6) and in 3 saline controls (Table 3). In both cases of uterine overdistention, fetal IL-1 β , TNF- α , IL-6, and IL-8 were markedly higher than in saline controls. In the 2 animals with uterine over-distention, fetal IL-6 levels met criteria for the fetal inflammatory response syndrome (>11 pg/mL).⁴⁶ In contrast, peak levels of maternal plasma cytokines (IL-1 β , TNF- α , IL-6, IL-8) were similar between controls and balloon cases.

Biomechanical modeling of uterine wall stress in the nonhuman primate model

To investigate the biomechanical effects of the varying balloon inflation protocols on uterine wall stress, we used the law of Laplace to approximate the relationship between intrauterine pressure and wall stress. We also took into account the small variations in size of the fetus and volume of amniotic fluid in each experiment to improve our estimates. In our calculations, we assumed the starting intrauterine pressures among the animals were the same. The calculated wall stress before balloon inflation differed at most by 5.4%, suggesting that large changes in wall stress after balloon inflation are not highly dependent on the initial resting pressure. After a brief equilibration period following balloon inflation, we calculated that pressures increased between 3.4 torr (animal no. 1) and 6.8 torr (animal no. 4) (Table 4). This translates to increases of uterine wall stress between 40-83%. From these calculations, it appears that some animals experienced a modest increase in uterine stress (animals no. 1, 2, and 6), and 3 animals experienced a much larger increase (no. 3, 4, and 5) (Table 4). The largest increase in uterine wall stress was associated with development of preterm labor in 2 of 3 cases.

Uterine and placental histopathology

Histopathology of uterine biopsies (lower uterine segment and fundus) and the placenta was reviewed to determine if balloon distention was associated with injury of either the myometrium or fetal membranes. Normal uterine tissue and placental histology was noted in all controls and balloon animals (data not shown). Rare individual perivascular neutrophils and lymphocytes were seen in uterine sections of both groups, without evidence of alteration of architecture or stromal changes. Placental histology was consistent with changes normally seen in late gestation without evidence of abruption or tissue necrosis. This suggests that labor in the balloon group was not the result of gross injury to the fetal membranes or myometrium.

Cytokine response in the amnion after in vitro stretch and in women with polyhydramnios

To determine if uterine stress was also associated with cytokine expression and secretion in vitro, we examined the longitudinal expression of labor-associated genes in human amniocytes up to 3 hours following stretch. IL1 β , IL6, and IL8 mRNA levels were significantly increased vs nonstretched controls at multiple time points following amniocyte stretch ($P < .05$) (Figure 2). There was no increase in TNF mRNA expression at any of the time points up to 6 hours after stretch. To further validate our observations, we investigated amnion tissues from women with polyhydramnios, in which acute (or subacute) uterine overdistention is thought to occur. Amnion tissues were obtained from women with polyhydramnios ($N = 3$) and preterm controls not in labor ($N = 36$) delivered by cesarean. There was a significant increase in mRNA expression of TNF ($P < .001$) and IL6 ($P < .05$), but not IL1 β or IL8 in the amnion from women with polyhydramnios (Figure 3). In summary, inflammatory cytokine mRNA expression is increased after stretch of human amniocytes in vitro and in the amnion of women with polyhydramnios, which supports the data observed in the nonhuman primate model.

Cytokine and chemokine protein levels in the amnion and myometrium of women with twins

To determine if inflammatory cytokine and chemokine proteins were increased in women with twins, we tested matched samples of amnion and myometrium from women with twins in early labor compared to twins not in labor. We chose twin pregnancies in early labor, because we hypothesized that these cases had crossed a threshold of distention necessary to trigger labor and would manifest a greater myometrial inflammatory response than twins not in labor at the same gestational age. TNF- α was significantly increased in the amnion of women with twins in early labor ($P < .01$) (Figure 4). In myometrial samples from women in early labor, IL-6, IL-8, and CCL2 levels were significantly elevated (all $P < .05$) (Figure 4). Both amnion and myometrial tissues contribute to inflammation in women with twins in early labor, but the cytokine profile from each tissue appears to be distinct.

Microarray, qRT-PCR validation, and gene set analysis

To explore changes in mRNA expression associated with balloon inflation in the monkey and development of pre-term labor, we performed a microarray on biopsies from the lower uterine segment at the time of cesarean delivery. We chose this uterine site, because studies have described more marked changes in myometrial gene expression associated with labor from the lower uterine segment vs the fundus.^{47,48} There was differential expression of 719 of 52,865 probe sets (449 up- and 270 down-regulated) at least 2.0-fold ($P < .05$) in the lower uterine segment after balloon inflation with development of preterm labor. A subset of these genes is shown in Table 5.

We identified 15 genes of interest significant in at least 1 of the contrasts from the microarray of the nonhuman primate myometrium, which we analyzed by qRT-PCR (Figure 5). There was a significant difference in lower uterine segment mRNA levels from balloon animals and controls for *HBEGF*, *EPHB1*, *MME*, *AGTR1*, and transforming growth factor, beta-induced (*TGFB1*) (all $P < .05$). To determine if similar genes were differentially expressed in human cases of over-distention, we performed qRT-PCR on myometrium from

the lower uterine segment of women with polyhydramnios and women with twins in early labor (Figure 6). In both women with polyhydramnios and twins in early labor, 4 of the 5 myometrial genes were differentially expressed in a similar direction as observed in the nonhuman primate model (*HBEGF*, *TGFBI* increased; *AGTRI* and *MME* decreased). Three of the 5 myometrial genes were significantly differentially expressed in either women with polyhydramnios (*TGFBI*) or both polyhydramnios and twins in early pre-term labor (*HBEGF*, *AGTRI*). These findings demonstrate remarkable overlap in gene expression across different species and different etiologies of uterine distention.

We performed IPA analysis to find known biological pathways that were overrepresented in our set of differentially expressed genes in the myometrium of our nonhuman primate model (IPA summary) (Table 6). “Cellular movement” was identified as a top “molecular and cellular functions” category, which contained 4 of the 5 genes validated by qRT-PCR in the nonhuman primate myometrium (*HBEGF*, *TGFBI*, *MME*, and *EPHB1*; IPA pathway $P = 3.21 \times 10^{-3} - 1.07 \times 10^{-8}$). The range of P values indicates that there are several subcategories within “cellular movement” category and the P values provide the range from the least to most significant for all subcategories. IPA identified several highly significant canonical pathways and predicted the transcription factors, hepatocyte growth factor and vascular endothelial growth factor,^{49,50} as upstream regulators that further supported cellular movement and migration as an important theme. High mobility group box-1 (*HMGB1*) signaling was identified as a significant canonical pathway; HMGB1 is a potent mediator of inflammation and induces migration of vascular smooth muscle cells (9.83×10^{-6}).⁵¹ “Regulation of cellular mechanics by calpain protease” was another significant canonical pathway; calpain proteases are a large family of intracellular proteases that act as key regulators of cell migration ($P = 3.78 \times 10^{-5}$).⁵²

To determine if uterine overdistention was associated with changes in uterine smooth muscle, IPA and gene set tests were used. In IPA, “skeletal and muscular system development and function” and “cardiovascular system development and function” were top “physiological system” categories ($P = 2.31 \times 10^{-3} - 8.45 \times 10^{-9}$ and $2.93 \times 10^{-3} - 3.63 \times 10^{-7}$, respectively). Using a self-contained gene set test, we identified 3 gene sets related to myogenesis and angiogenesis that we predicted would be differentially regulated after uterine stretch associated with labor (gene ontology biological process “angiogenesis” and “muscle development,” Reactome “myogenesis” pathway). All 3 gene sets were highly significant suggesting that uterine overdistention was associated with an effect on muscle growth and angiogenesis (muscle development, $P = .003$; angiogenesis, $P = .002$; myogenesis, $P = .001$).

Comment

Principal findings of the study

Principal findings of the study were as follows. (1) Preterm labor occurred in 3 of 6 animals after balloon inflation and correlated with greater balloon volume and uterine wall stress. (2) Significant elevations of inflammatory cytokines and prostaglandins occurred following uterine overdistention in an inflammatory pulse that correlated with preterm labor. (3) A similar inflammatory response was observed in amniocytes in vitro following mechanical

stretch, in amnion of women with polyhydramnios, and in amnion and myometrium of women with twins in early labor. (4) Genes differentially expressed in the nonhuman primate after balloon inflation and in women with polyhydramnios and twins are involved in tissue remodeling and muscle growth.

Preterm labor correlated with greater balloon volume and myometrial stress

Preterm labor rates are greater in women with multiple pregnancy and larger singleton babies, suggesting that labor can be initiated by exceeding a critical uterine capacity.⁵³ However, even in these cases, the variation in gestational age at delivery suggests that the ability of the human uterus to accommodate overdistention is modulated by other factors. Our data show that preterm labor in the macaque correlated with greater balloon volume; this supports the hypothesis that there is a threshold for amnion or myometrial stress, where below threshold, volume increases can be accommodated, but above threshold, labor is initiated. Similar to human beings, the macaque uterus appeared to handle increasing uterine wall stress differently among individuals.⁵⁴ There are clearly a number of variables that cannot be fully controlled that directly influence an individual's response. One of those factors is the potential to reduce amniotic fluid volume before mechanical stimulation can affect gene expression. However, it is important to consider how mechanical stress on the uterine wall effects myometrial and amniotic stresses differently.

In some individuals, the myometrium may more readily elongate (creep) in response to the pressure rise caused by balloon inflation. Enhanced myometrial creep will reduce intrauterine pressure and the long-term stress on the myometrium, but it will acutely increase the stress on the amnion since creep increases the surface area of the uterine wall. In contrast, the myometrium of other individuals may resist creep, and instead maintain a greater intrauterine pressure. In this case the surface area of the uterus is relatively unchanged, resulting in greater stress on the myometrium but less change in amnion stress. Overall, individual variations can be seen to result in mechanical changes that effect primarily myometrium, primarily amnion, or a mixture of each. Indeed, a key limitation in our analysis of the mechanical properties of the uterus is the gap in knowledge regarding the nonlinearity of the stress-strain relationship and the mechanical limits of amnion and myometrium at various gestations and in different individuals.

Elevations in cytokines and prostaglandins occurring following uterine overdistention in an inflammatory pulse that correlated with preterm labor

Our results suggest a novel inflammatory mechanism for preterm labor associated with uterine overdistention that occurred as a cytokine and prostaglandin pulse, which differs from infection-associated preterm labor in which cytokines and prostaglandins tend to increase in a logarithmic scale.²¹ A larger cytokine pulse correlated with greater balloon volume, myometrial stress, and a more profound stimulus on myometrial gene expression. Serial sampling of the amniotic fluid was key for detection of the inflammatory pulse, which is not possible in pregnant women or many other animal models. The source of the amniotic fluid inflammatory cytokine pulse may come from the fetal membranes and/or the myometrium. Fetal cytokines were also markedly elevated in 2 animals with uterine

overdistention, but this result was difficult to interpret due to the potential impairment of placental/umbilical blood flow by the balloons.

Inflammation occurred in amniocytes following mechanical stretch and in amnion and myometrium of women with polyhydramnios and twins in early labor

Myometrial inflammation is a well-recognized feature of term labor.^{55,56} However, a role for inflammation in the onset of uterine overdistention has not been identified to date, although in vitro studies have shown that stretch of both amnion and myometrial cells results in the release of cytokines and chemokines.^{17,45} Our results are consistent with these prior studies, but now demonstrate significant cytokine increases in the amnion of women with polyhydramnios (TNF- α and IL-6 mRNA) and in the amnion (TNF- α) and myometrium of women with twins in early preterm labor (IL-6, IL-8, and CCL2). Elevation of CCL2 in nonhuman primate amniotic fluid and twin myometrium is also consistent with the rat model of overdistention and stretch of cultured human myometrial cells.^{17,57} The inflammatory response may also differ between amnion and myometrial tissues suggesting that both contribute to parturition, but possibly through different cytokines/chemokines and kinetics. Individual susceptibility to labor is likely a complex combination of multiple factors including genetic propensity toward a hyperinflammatory response, as well as hormonal milieu, intrauterine pressure, and uterine wall thickness.

Genes differentially expressed in the nonhuman primate after uterine overdistention and in women with polyhydramnios and twins are involved in tissue remodeling and muscle growth

Analysis of differentially expressed genes and signaling pathways suggested that key genes and pathways involved in cell migration (tissue remodeling) and muscle development were strongly associated with uterine overdistention in the nonhuman primate as well as in women with polyhydramnios and twins. Tissue remodeling is common in many other cell types following mechanical stress and the resulting inflammatory response (vascular smooth muscle,⁵⁸⁻⁶⁰ endothelium,⁶¹⁻⁶³ colonic smooth muscle,⁶⁴ endometrial stromal cells,⁶⁵ lung epithelium⁶⁶). Extensive remodeling of myometrial smooth muscle and the extracellular matrix is required for the onset of labor.^{67,68} HBEGF may be a key gene linking uterine overdistention with preterm labor, inflammation, and myometrial tissue remodeling; HBEGF has been identified as up-regulated in stretch of the human bladder,⁶⁹ spontaneous labor at term,⁷⁰ cell migration and tissue remodeling,^{71,72} and as a chemotactic factor in cancer cells.⁷³ Up-regulation of TGFBI also acts to inhibit cell adhesions, thereby increasing cell motility.⁷⁴ Additional evidence for tissue remodeling came from the identification by IPA of activated canonical pathways (HMGB1, calpain proteases) and transcription factors (hepatocyte growth factor) classically associated with cell migration. Our results are also consistent with findings from a rat balloon model of overdistention linking mechanical stress with myometrial smooth muscle growth and proliferation.^{57,75} The link between uterine overdistention and muscle growth and development represents a logical pairing of mechanical signals with preparation for labor or perhaps a compensatory mechanism allowing the myometrium to accommodate the uterine strain.

The strength of our study is that it is the first study of excessive uterine over-distention in a nonhuman primate model, which is very similar to human pregnancy. Preterm labor in women with multiple gestation or acute polyhydramnios is challenging to investigate for several reasons. First, biomechanical changes in uterine strain or stretch are difficult to isolate from other mechanisms that might induce preterm labor. It has been hypothesized that late PTB in women with a multiple gestation occur in part due to a double dose of fetal hormones deriving from 2 fetuses, with each producing hormones in the fetal hypothalamic-pituitary-adrenal-placental axis contributing to parturition. Secondly, women with a multiple gestation have a higher incidence of short cervix and preterm premature rupture of membranes, which may be attributed to infection. Finally, biological samples collected at the time of preterm labor, regardless of the initial mechanistic trigger, are biologically somewhat similar with high levels of inflammatory mediators in a common final pathway. Overall, our nonhuman primate model better isolates uterine strain from other mechanisms of parturition than prior studies in human beings and allows for serial sampling to identify temporal patterns in biological mediators.

Our model may be more similar to polyhydramnios in which an acute increase in uterine volume and wall tension tends to occur rather than multiple gestation in which volume increases tend to occur over a longer time period. Whether an increase in uterine wall tension might contribute to PTB in women with multiple gestation is controversial. A previous assessment of the relationship between uterine wall structure and tension in pregnant women concluded that wall tension was not increased in twins compared to singleton pregnancies.²⁸ However, this conclusion hinged on the observations of a prior study³⁰ that measured intraamniotic pressures using amniocentesis and found no differences between singletons and twins. It is difficult to use these data to address the relationship between intrauterine volume and the onset of labor in twins for several reasons. First, the study of intraamniotic pressures³⁰ was not sufficient to fully define the relationship between singleton and twin resting intrauterine pressures, which is key to understanding wall tension at any gestation. Second, the study of wall tension in twins measured wall thickness in only 11 twin pregnancies; precise measurements over multiple regions of the uterus obtained at similar gestational ages across subjects would be required to fully determine the small degree of wall thinning necessary to increase wall stress. Third, to fully determine the contribution of the increased volume in twin pregnancies at each gestational age, it would be also necessary to assess how the uterus accommodates dynamic change associated with advancing pregnancy. The relationship among multiple gestation, wall stress, and advancing gestation is complicated and not well defined in the literature.

A key study limitation is the small sample size of our nonhuman primate study, which is a necessary constraint of primate research due to ethics and expense of the work. Repeating our saline control experiments to allow for randomization of controls and balloon animal experiments was not possible for ethical and funding reasons. Another limitation is that we ended our slow-stretch experiments after only a few weeks, which means that we did not determine if labor might have occurred somewhat earlier than term (eg, late PTB) in these animals with expectant management. It is also possible that increased wall stress from an expanding uterine volume might have impeded placental blood flow, which represents an unknown variable influencing our results and possibly fetal cytokine levels. Interestingly,

impaired placental blood flow in the setting of incomplete remodeling of the spiral arteries (defective deep placentation) is also correlated with PTB and may also have an inflammatory basis.⁷⁶⁻⁷⁸ The effect of fetal demise occurring in 2 of the 3 animals developing preterm labor is unknown, but a similar pattern of an inflammatory pulse occurring after balloon inflation occurred in the animals with surviving fetuses and is known to occur in myometrium after an experimental stretch *ex vivo* and *in vitro*.^{16,79} Small sample size is a limitation of our polyhydramnios cohort, which reflects the rarity of the diagnosis; we present results from these women, because the condition may reflect a model of uterine overdistention with fewer confounding factors. Labor is itself an inflammatory condition and the presence of early labor in our twin cohort may have confounded our interpretation of the results; we present these results because we hypothesized that inflammation was the driving factor underlying labor in these twins and limited consideration to only cases in very early labor.

Overall, our model provides a unique opportunity to understand how uterine overdistention and biomechanical forces on the uterus induce preterm labor. Future research in the nonhuman primate model could address the question of whether slower inflation might sufficiently destabilize uterine quiescence over time to result in a late PTB. Additional soluble effectors and genes associated with this process remain to be defined and include previously implicated mediators of uterine stretch and labor including chemokines (eg, CCL2) and genes involved in smooth muscle and extracellular matrix remodeling. Finally, we are encouraged that labor associated with uterine overdistention might be mediated by inflammation, which suggests potential therapeutic targets to prolong pregnancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. Temporal relationships among balloon inflation, cytokines and uterine activity

Temporal relationships among intraamniotic balloon inflation, amniotic fluid cytokines, and uterine activity. **A**, Saline inoculation into amniotic fluid and choriodecidual space days 0 and 4. No balloons were inflated and labor did not occur by day 7. **B**, Intraamniotic balloon inflation with labor. Two balloons were inflated to maximum balloon inflation of 154 mL over 4 days. “Inflammatory pulse” followed balloon inflation and preceded labor on day 8. **C**, Intraamniotic balloon inflation without labor. Two balloons were inflated to maximum

balloon inflation of 160 mL over 18 days. No labor by day 18. With greater volumes of balloon inflation at end of experiment, there is rise in IL-8.

IL, interleukin; *PGE₂*, prostaglandin E₂; *PGF_{2α}*, prostaglandin *F_{2α}*; *TNF*, tumor necrosis factor.

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FIGURE 2. Messenger RNA (mRNA) levels after amniocyte stretch

Levels of **A**, IL1, **B**, TNF α , **C**, IL6, and **D**, IL8 mRNA were measured by real-time quantitative reverse transcriptase polymerase chain reaction in amniocytes after 11% stretch (N = 8). Results are corrected for glyceraldehyde 3-phosphate dehydrogenase expression and given as ratio to nonstretched samples (*P < .05). Results are shown as mean with SEM (N = 8).

IL, interleukin; TNF, tumor necrosis factor.

FIGURE 3. Messenger RNA (mRNA) levels in amnion from women with polyhydramnios Levels of **A**, IL1 β , **B**, TNF α , **C**, IL6, and **D**, IL8 mRNA were measured by real-time quantitative reverse transcriptase polymerase chain reaction in amnion cells of women with polyhydramnios (N = 3) vs women delivering preterm not in labor (N = 36). Results are corrected for glyceraldehyde 3-phosphate dehydrogenase expression and are shown as median with range (* $P < .05$; *** $P < .001$; $P < .0001$). Note that there was 1 less amnion sample available from woman with polyhydramnios for this experiment than in Figure 6. *IL*, interleukin; *TNF*, tumor necrosis factor.

FIGURE 4. Amnion and myometrium cytokine levels in women with twins

Protein levels of inflammatory cytokines and chemokines in amnion and myometrial tissues of women with twins in early labor vs not in labor (A, C, E, G, and I: amnion; B, D, F, H, and J: myometrium; N = 9, twins in early labor; N = 15, twins not in labor). Results are shown as median with range (* $P < .05$; ** $P < .01$).

CCL, chemokine (C-C motif) ligand; *IL*, interleukin; *TNF*, tumor necrosis factor.

FIGURE 5. Quantitative reverse transcriptase PCR validation of differentially expressed myometrial genes

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) validation of microarray results from nonhuman primate myometrium. X-axis represents individual genes and y-axis, fold-change in expression of difference between balloon inflation cases and saline controls. All genes shown were significant in unadjusted microarray analysis. *Genes significantly up- or down- regulated by qRT-PCR (2-sided *t* test, $P < .05$).

AGTR1, angiotensin II receptor type 1; *CCL2*, chemokine (C-C motif) ligand 2; *EPHB1*, ephrin type-B receptor 1; *HBEGF*, heparin-binding epidermal growth factor-like growth factor; *MME*, membrane metalloendopeptidase; *TGFB1*, transforming growth factor, beta-induced.

FIGURE 6. Quantitative reverse transcriptase PCR on myometrium from women with polyhydramnios and twins

Quantitative reverse transcriptase polymerase chain reaction performed to quantitate messenger RNA levels in myometrium from women with polyhydramnios (N = 4, polyhydramnios; N = 10, singleton gestational age-matched controls not in labor) and twins (N = 9, twins early labor; N = 12, twins not in labor). Levels of *AGTR1* (A, polyhydramnios; B, twins), *EPHB1* (C, polyhydramnios; D, twins), *HBEGF* (E, polyhydramnios; F, twins),

MME (**G**, polyhydramnios; **H**, twins), and *TGFI* (**I**, polyhydramnios; **J**, twins) were measured. Results are shown as median with range (* $P < .05$; ** $P < .01$). *AGTRI*, angiotensin II receptor type 1; *EPHBI*, ephrin type-B receptor 1; *HBEGF*, heparin-binding EGF-like growth factor; *MME*, membrane metalloendopeptidase; *TGFI*, transforming growth factor, beta-induced.

TABLE 1

Intraamniotic balloon inflation protocols and outcomes

Experiment no.	<u>Intraamniotic balloons</u>			Days to maximum inflation	Intrapartum fetal demise	Labor within 10 d	Days to delivery
	No.	Total volume, mL					
1	2 ^a	160		18	No	No	18
2	2	154		4	No	No	11
3	3	246		2	No	No	39
4	3	246		1	Yes	Yes	3
5	2	224		2	Yes	Yes	4
6	2	154		4	No	Yes	8

^aOne balloon was found deflated at time of cesarean delivery.

TABLE 2

Amniotic fluid cytokines and prostaglandins

Analyte	Preinflation		Postinflation			P value
	Saline infusion (N = 5)	Balloon inflation (N = 6)	Saline infusion	Balloon inflation, no labor (N = 2) ^a	Balloon inflation, labor (N = 3)	Saline vs balloon, labor
IL-1 β	0.003 (0.001)	0.03 (0.02)	0.005 (0.002)	1.3 (1.1)	3.7 (3.3)	.003
TNF- α	0.01 (0.009)	0.02 (0.008)	0.02 (0.01)	0.4 (0.3)	0.9 (0.4)	.02
IL-6	4.3 (1.4)	8.4 (3.7)	10.7 (2.2)	28.4 (21.2)	78.3 (25.9)	.02
IL-8	0.8 (0.2)	8.9 (3.8)	1.5 (0.3)	13.6 (10.2)	26.9 (2.4)	.001
CCL2	1.4 (1.0)	1.9 (0.9)	3.0 (2.0)	5.3 (3.8)	9.4 (3.5)	.007
PGE ₂	0.7 (0.6)	0.09 (0.02)	0.7 (0.5)	0.4 (0.3)	1.3 (0.4)	.001
PGF _{2α}	0.3 (0.06)	0.3 (0.09)	0.7 (0.1)	0.7 (0.2)	2.9 (1.2)	<.001

Table presents mean (SEM). Values for amniotic fluid analyses are ng/mL. *P* values are based on analysis of covariance models adjusting for baseline. Saline infusion was performed in both amniotic fluid and choriodecidual compartments. Balloon inflation was performed within amniotic cavity.

CCL, chemokine (C-C motif) ligand; *IL*, interleukin; *PGE*₂, prostaglandin E₂; *PGF*_{2 α} , prostaglandin F_{2 α} ; *TNF*, tumor necrosis factor.

^aNo amniotic fluid could be obtained from 1 animal undergoing balloon inflation with no labor, reducing N to 2 in this group.

TABLE 3 Fetal cytokines

Experiment no.	Fetal plasma cytokines ^a			
	IL-1 β	TNF- α	IL-6	IL-8
Saline 1	1.2	1.2	2.0	523.4
Saline 2	1.2	1.2	1.2	182.3
Saline 3	1.2	2.2	2.4	223.0
Balloon 2	27.2	90.3	22.8	4014.4
Balloon 6	3	14.8	14	927

IL, interleukin; TNF, tumor necrosis factor.

^aValues are pg/mL. Peak values for saline controls and values at delivery for uterine overdistention animals. Fetal cytokines were only available from 3 saline controls and 2 animals undergoing uterine overdistention. Balloon animals no. 2 and 6 correspond to same experiments numbered in Tables 1, 2, and 4.

TABLE 4

Intrauterine pressure and wall stress associated with balloon inflation

	<u>Before balloon inflation</u>			<u>After balloon inflation</u>				
	<u>Intrauterine pressure</u>	<u>Wall stress</u>	<u>Deviation from average wall stress</u>	<u>Intrauterine pressure</u>	<u>Pressure increase</u>	<u>Wall stress</u>	<u>Increase wall stress</u>	
Experiment no.	Pa (torr) ^a	N/m ² × 10 ³	%	Pa (torr) ^a	%	N/m ² × 10 ³	%	Labor within 10 d
1	1600 (12)	134	5.4	2049 (15.4)	28	187	40	No
2	1600 (12)	128	1.1	2112 (15.8)	32	186	45	No
3	1600 (12)	127	0	2398 (18.0)	50	220	73	No
4	1600 (12)	123	−3.2	2500 (18.8)	56	224	83	Yes
5	1600 (12)	123	−2.8	2420 (18.2)	51	215	75	Yes
6	1600 (12)	126	−0.5	2145 (16.1)	34	187	48	Yes

Experiment number in Tables 1 and 3 correspond to same animals to allow for comparison across tables.

^aPa is an International System of Units measure of pressure and stress defined as 1 N/m²; torr is a unit of pressure that is approximately equal to 1 mm Hg and is presented as this may be more intuitive for clinician.

TABLE 5

Subset of differentially expressed genes from lower uterine segment of balloon animals developing preterm labor and saline controls

Probe ID	Entrez ID	Symbol	Log2 fold change	P value
MmugDNA.34986.1.S1_at	717623	HLA-DQA1	2.75	.03
MmugDNA.34073.1.S1_at	717912	MYH2	2.32	.01
MmuSTS.3317.1.S1_at	574138	CCL2	2.28	.005
MmuSTS.2130.1.S1_s_at	714690	S100A9	2.24	.06
MmuSTS.4661.1.S1_at	701850	TNFRSF11B	2.04	.01
MmugDNA.17376.1.S1_at	714740	S100A8	2.04	.07
MmuSTS.3545.1.S1_at	574218	CCL11	1.85	.02
MmugDNA.28443.1.S1_at	696285	SMPX	1.76	.003
MmugDNA.16956.1.S1_s_at	705000	MYOZ2	1.68	.01
MmuSTS.4072.1.S1_at	695559	HBEGF	1.37	.0002
MmugDNA.334.1.S1_at	701805	SERPINB2	1.36	.02
MmugDNA.18704.1.S1_at	712773	AGTR1	-1.29	.006
MmugDNA.22309.1.S1_at	717211	EPHB1	-2.00	.0007
Mmu.1481.2.S1_s_at	707667	MME	-2.75	.0004
Mmu.5303.1.S1_at	720582	MAMU-DQA1	-4.26	.04
MmugDNA.2178.1.S1_s_at	699243	MAMU-A3	-6.26	.0009

AGTR1, angiotensin II receptor type 1; *CCL2*, chemokine (C-C motif) ligand 2; *EPHB1*, ephrin type-B receptor 1; *HBEGF*, heparin-binding epidermal growth factor-like growth factor; *MME*, membrane metalloendopeptidase.

TABLE 6

Ingenuity pathway analysis summary

Functional analysis of network ^a		
	No. of molecules	P value
<i>Diseases and disorders</i>		
Cancer	125	$3.21 \times 10^{-3} - 9.40 \times 10^{-14}$
Organismal injury and abnormalities	77	$2.75 \times 10^{-3} - 6.44 \times 10^{-9}$
Reproductive system disease	60	$2.65 \times 10^{-3} - 6.44 \times 10^{-9}$
<i>Molecular and cellular functions</i>		
Cellular development	75	$2.93 \times 10^{-3} - 8.45 \times 10^{-9}$
Cellular growth and proliferation	78	$2.93 \times 10^{-3} - 8.45 \times 10^{-9}$
Cellular movement	58	$3.21 \times 10^{-3} - 1.07 \times 10^{-8}$
<i>Physiological systems</i>		
Skeletal and muscular system development and function	28	$2.31 \times 10^{-3} - 8.45 \times 10^{-9}$
Tumor morphology	40	$2.68 \times 10^{-3} - 1.27 \times 10^{-7}$
Cardiovascular system development and function	49	$2.93 \times 10^{-3} - 3.63 \times 10^{-7}$
Canonical pathway analysis ^b		
Top canonical pathways	Ratio	P value ^d
Hepatic fibrosis/hepatic stellate cell activation	11/155	4.73×10^{-7}
HMGB1 signaling	8/109	9.83×10^{-6}
Regulation of cellular mechanics by calpain protease	6/73	$6/73 \ 3.78 \times 10^{-5}$
Upstream regulator analysis ^c		
Top transcription factors	Predicted activation state	P value of overlap
F2	Activated	7.77×10^{-13}
HGF	Activated	6.25×10^{-10}
VEGF	Activated	9.72×10^{-10}

HGF, hepatocyte growth factor; HMGB1, high mobility group box-1; VEGF, vascular endothelial growth factor.

^aIdentified biological functions and/or diseases that were overrepresented among molecules in network using right-tailed Fisher exact test

^bIdentified pathways from Ingenuity Pathway Analysis library that were most significant to data set. Significance of association was measured in 2 ways: (1) as ratio of number of molecules from focus gene set that map to pathway to total number of molecules that map to canonical pathway; and (2) using Fisher exact test

^cBased on prior knowledge of expected effects between transcription factors and their target genes stored in Ingenuity Pathway Analysis library. Overlap P value measures whether there is statistically significant overlap between data set genes and genes regulated by transcription factor using Fisher exact test

^dRange of P values indicates that category listed in table contained several subcategories. P values provide range from least to most significant for all subcategories.