Anti-inflammatory effects of gallic acid in human gestational tissues *in vitro*

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Abstract

Spontaneous preterm birth is the leading cause of neonatal mortality and morbidity globally. Activation of the maternal immune system leads to a downstream cascade of proinflammatory events that culminate in the activation of spontaneous uterine contractions and the rupture of the foetal membranes. Anti-inflammatory agents may be a novel therapeutic approach to prevent inflammation-induced myometrial contractions and premature rupture of foetal membranes. The polyphenol gallic acid has been previously shown to exert potent anti-inflammatory effects. Thus, this study aimed to determine the effect of gallic acid on proinflammatory and pro-labour mediators in cytokine-stimulated gestational tissues *in vitro*. In primary human cells isolated from myometrium and foetal membranes (decidua, and amnion mesenchymal and epithelial cells), gallic acid treatment suppressed inflammation-induced expression of proinflammatory cytokines and chemokines and extracellular matrix-degrading and matrix-remodelling enzymes. Gallic acid also significantly inhibited inflammation-induced myometrial activation as evidenced by decreased expression of contraction-associated proteins, the uterotonic PGF_{2α} and collagen cell contractility. Using a global proteomic approach, gallic acid may differentially regulate proteins associated with collagen synthesis, cell contractility and protein synthesis in primary myometrial and decidual cells. In summary, gallic acid inhibited inflammation-induced mediators involved in active labour in primary cells isolated from myometrium and foetal membranes. These *in vitro* studies suggest that the polyphenol gallic acid may be able to suppress the production of proinflammatory and pro-labour mediators involved in myometrial contractions and rupture of foetal membranes. Future preclinical studies may elucidate the efficacy of gallic acid in preventing inflammation-driven preterm birth.

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Introduction

Spontaneous preterm birth is a global health issue affecting 15 million pregnancies every year (Beck *et al.* 2010). The most critical consequence is neonatal death, accounting for 1 million neonatal deaths worldwide (Blencowe *et al.* 2012). Survivors of preterm birth also face significant long-term neurological morbidity, including cerebral palsy, developmental delay, and hearing and visual impairment (Mwaniki *et al.* 2012).

Spontaneous preterm birth shares the common terminal pathways with normal parturition, including uterine activation and rupture of membranes. The triggers, however, differ with infection and

inflammation associated with half of all preterm births (Goldenberg *et al.* 2000). Bacterial endotoxins or proinflammatory insults activate the maternal immune system to drive leukocyte infiltration into the gestational tissues (Thomson *et al.* 1999, Hamilton *et al.* 2012). The infiltrating immune cells then induce production of proinflammatory cytokines, such as interleukin (IL)-1B and TNF, which further propagate inflammation (Lappas *et al.* 2006, Lappas 2017) and production of other pro-labour mediators (Rauk & Chiao 2000, Kumar *et al.* 2006). Inflammation induces contraction-associated proteins, uterotonics, and extracellular matrix (ECM) degrading and remodelling enzymes, ultimately resulting in myometrial contractions and

foetal membrane rupture associated with spontaneous preterm birth (Vadillo-Ortega & Estrada-Gutierrez 2005, Olson & Ammann 2007). Similarly, oxidative stress is present in the foetal membranes (Chai *et al.* 2012) and is thought to contribute to the development of pPROM and preterm birth (Moore *et al.* 2018). Unfortunately, current interventions provide limited assistance for inflammation-driven preterm birth. Progesterone is effective only in women with a short cervix, while tocolytic therapies delay preterm birth for only 48 h and present little neonatal benefit (Haas *et al.* 2012). Antibiotic use is also effective for women with pPROM (Kenyon *et al.* 2013); however, little evidence exists to demonstrate an effect of antibiotics against myometrial contractions in preterm labour (Flenady *et al.* 2013).

Polyphenols are natural, plant-based compounds that have demonstrated anti-inflammatory properties and are categorised into four key groups, including flavonoids, stilbenes, lignans and phenolic acids (Manach *et al.* 2004). Our previous studies have shown that polyphenols belonging to the flavonoids (nobiletin, naringenin, apigenin, silibinin, luteolin and kaempferol), lignans (honokiol) and phenolic acids (curcumin) can suppress inflammation-induced expression of proinflammatory cytokines, chemokines, prostaglandins and ECM-degrading enzymes in placenta, myometrium and foetal membranes (Lim *et al.* 2013c, 2014, Wall *et al.* 2013, Morwood & Lappas 2014, Wijesuriya & Lappas 2018). Notably, recent epidemiological studies demonstrate that plant-based diets, and specifically high consumption of raisins, are associated with reduced incidence of preterm birth (Myhre *et al.* 2013, Englund-Ogge *et al.* 2014). Raisins are a rich source of the phenolic acid gallic acid (Shao *et al.* 2016) which possesses potent anti-inflammatory properties in several laboratory (Choi *et al.* 2009, Lee *et al.* 2015) and animal models of inflammation (Hsiang *et al.* 2013, Pandurangan *et al.* 2015). There are no studies, however, that have investigated the anti-inflammatory potential of the phenolic acid gallic acid in human gestational tissue in the context of spontaneous preterm birth.

In this study, we sought to determine the effect of gallic acid on: (1) proinflammatory cytokines and chemokines, (2) myometrial cell contractility and the prostaglandin pathway, and (3) ECM-degrading and ECM-remodelling enzymes. To assess this, we used primary human cells isolated from pregnant non-labouring myometrium and foetal membranes (decidua, amnion mesenchymal and amnion epithelial), and incubated the cells with the proinflammatory cytokines IL1B or TNF to generate an *in vitro* model of intrauterine inflammation. Myometrial cells and decidual cells were also used for label-free quantitative mass spectrometry and bioinformatics analysis to identify potential signalling pathways through which gallic acid may exert its effects.

Methods and materials

Human tissue collection

Ethics approval was obtained from the Mercy Hospital for Women's Research and Ethics Committee (Mercy Health, Ethics approval number R04-29) and written informed consent was obtained from all participating subjects. Myometrium and foetal membranes were obtained from healthy women with a BMI <30 kg/m² on the day of term Caesarean section (37–41 weeks of gestation). All samples were obtained from women who had no clinical signs of infection. Indications for Caesarean section in the absence of labour were breech presentation and/or previous Caesarean section. The following exclusion criteria were applied: vascular/renal complication, multiple gestations, asthma, smokers, preeclampsia, chorioamnionitis, placental abruption, acute foetal distress and women with any other adverse underlying medical conditions. All tissues were processed within 15 min of delivery.

Primary cell culture

Myometrial cells (Lim *et al.* 2013a), amnion epithelial and mesenchymal cells (Lim & Lappas 2019) and decidual cells (Araujo *et al.* 2018) were isolated and cultured as previously described. Cells were cultured in DMEM/F-12 containing 100 U/mL penicillin, 100 µg/mL streptomycin and 10% NBS (for myometrial cells) or 10% FBS (for amnion and decidual cells), until they reached 80% confluency. Cells were then split into 48-well tissue culture plates and at approximately 80% confluence were treated with 100 µM gallic acid or vehicle control (0.1% (v/v) DMSO) with or without 1 ng/mL IL1B or 10 ng/mL TNF for 20 h. The optimised dose of gallic acid was based on previous studies in other cells (Hsiang *et al.* 2013, Pandurangan *et al.* 2015) and a dose–response test (data not shown). After a 20-h incubation, cells were collected and stored at –80°C for analysis of pro-labour mediators by RT-quantitative PCR (RT-qPCR) (*n*=6 patients) or quantitative mass spectrometry (*n*=6 patients) as detailed subsequently. The incubation medium was collected and stored at –20°C for analysis of pro-labour mediators by ELISA (*n*=6 patients) or gelatin zymography (*n*=5 patients).

ELISA

Media from the tissue explants were assayed using a sandwich ELISA to determine the protein concentrations of IL6, GM-CSF and CXCL8 (Life Technologies) and IL1A, IL1B, CCL2, CXCL1 and CXCL5 (R&D Systems) as according to the manufacturer's instructions. The release of PGF_{2α} into the incubation medium was also assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's instructions (Cayman Chemical Company). The

calculated interassay and intraassay coefficients of variation (CV) were all less than 10%.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from tissues and converted to cDNA as previously described (Wijesuriya & Lappas 2018). The cDNA was diluted to 1 ng/μL in sterile milli-Q water. The SensiFAST™ SYBR No-ROX Kit (Bioline, Alexandria, NSW, Australia) and 100 nM of pre-designed and validated primers (Qiagen) were used to perform RT-PCR on CFX384 Real-Time PCR detection system from Bio-Rad Laboratories (Gladesville, NSW, Australia). Average gene Ct values were normalised against the housekeeping genes succinate dehydrogenase complex subunit A (SDHA) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ). There was no effect of experimental treatment on SDHA or YWHAZ mRNA expression. Fold differences were determined using the comparative Ct method.

Collagen gel contraction assay

Myometrial cell contraction assays were performed as previously described (Wijesuriya & Lappas 2018). Primary human myometrial cells (isolated as detailed above) were resuspended in 0.25 mL DMEM/F12 (containing 10% NBS) and mixed with 40 μL collagen (3 mg/mL collagen I from rat protein solution; Gibco™) and 1 μL 1 M NaOH by gently pipetting. The mixture was transferred to 48-well tissue culture plates, incubated at 37°C for 30 min to allow polymerisation and then treated with 10 ng/mL TNF with or without 100 μM gallic acid. The gel matrix was gently detached from the well, incubated for 50 h at 37°C, and the area of the gel was determined using Image Lab 3.0 (Bio-Rad Laboratories). Experiments were performed from myometrium obtained from five patients.

Gelatin zymography

Media was used to assess matrix metalloproteinase (MMP) 9 activity using gelatin zymography as previously described (Lim *et al.* 2013b). Proteolytic activity was visualised as clear zones of lysis on a blue background of undigested gelatin. Gels were scanned using a ChemiDoc XRS system (Bio-Rad Laboratories), and densitometry was performed on inverted images using Image Lab 3.0 (Bio-Rad Laboratories).

Quantitative mass spectrometry

Mass spectrometry analyses were conducted within an ISO17025 accredited (National Association of Testing Authorities, Australia) research facility, as previously described (Jayabalan *et al.* 2019). Protein extraction was performed on human primary myometrial and

decidual cells using 25 μL radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma) supplemented with protease inhibitor (Roche). After sonication for 5 min, the resulting cell lysates were clarified by centrifugation at 8000 g for 10 min. The protein concentrations were estimated using a bicinchoninic acid assay kit according to the manufacturer's instruction (Thermo). *Filter Aided Sample Preparation*: The cell lysates were processed for mass spectrometry using the filter aided sample preparation (FASP) method (Barr *et al.* 2011). In brief, 8 μg of proteins from each cell lysate sample were diluted with urea buffer (8 M urea, 100 mM Tris, pH 8.5). Samples were transferred into a Nanosep® filter unit with a 30 K molecular weight cut off and centrifuged for 10,000 g for 15 min and flow-through discarded. Proteins were reduced with 100 mM dithiothreitol, washed with urea buffer and alkylated with 50 mM iodoacetamide. The samples were equilibrated with 50 mM ammonium bicarbonate and digested using 1 μg of trypsin overnight at 37°C. *Desalting*: The tryptic peptides from individual samples were desalted with 0.1% (v/v) trifluoroacetic acid (TFA) in water using SOLAμ HRP SPE 96-well plate (Thermo Fisher Scientific) according to the manufacturer's instructions. *Analysis of peptides*: The peptides were then processed in an information-dependent acquisition (IDA) manner on an AB Sciex 5600 TripleTOF mass spectrometer. For SWATH acquisition on individual samples, the TripleTOF® 5600 System was configured as described by Gillet *et al.* (Catalano & Ehrenberg 2006). *Data Processing*: To generate the local ion library, a protein database search was conducted using the ProteinPilot version 4.5b Software (AB SCIEX) and the Paragon™ Algorithm. The search was performed against the SwissProt *Homo sapiens* database with a global false discovery rate (FDR) of 1% used as the threshold for the number of proteins for import. The SWATH Acquisition Microapp 2.0 in PeakView 2.2 (SCIEX) was used to process raw SWATH data files. Processing settings for the SWATH Microapp include two peptides per protein, three transitions per peptide, peptide confidence threshold corresponding to 1% global FDR and FDR threshold of 1%. The resulting peak area for each protein after SWATH processing was exported to MarkerView 1.3.1 (SCIEX) for statistical analysis and the resulting data normalised using the Total Area Sums (TAS) approach.

Ingenuity pathway analysis (IPA)

Pathway enrichment analyses were performed with Ingenuity Pathway Analysis (IPA, Qiagen) as we previously described (Nguyen-Ngo *et al.* 2020). IPA was performed to identify canonical pathways, diseases and functions, and protein networks associated with significantly changed proteins. Statistical significance of enriched pathways for the proteins and pathways were denoted with *P*-value <0.05.

Statistical analysis

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software). Shapiro–Wilk test was used to test the normality of all the data. Data for Figs 1, 2, 3, 4, 5, 6, 7, 8 and 9 were analysed by a repeated measures one-way ANOVA (with LSD post hoc testing to discriminate among the means); data for Fig. 10 was analysed by Student's *t*-test; non-normally distributed data were logarithmically transformed before analysis. Statistical significance was ascribed to a *P*-value ≤ 0.05 . Data were expressed as mean $\pm$ S.E.M.

Results

Effect of gallic acid on proinflammatory cytokines in myometrial, decidual and amnion cells

The effect of gallic acid on proinflammatory cytokine expression and secretion in primary human myometrial, decidual and amnion mesenchymal cells is shown in Figs 1, 2 and 3. For all cells, IL1B-induced IL1B mRNA expression and TNF-induced GM-CSF secretion were non-detectable. Further, IL1A and IL1B levels in the incubation media were also below the sensitivity of the assay for all cells. In amnion epithelial cells, for the majority of the patients, IL6 and CCL2 levels in the incubation media of amnion epithelial cells were also below the sensitivity of the assay. Thus, no further inflammatory analysis was conducted on amnion epithelial cells.

IL1B or TNF treatment significantly increased IL1A, IL1B, IL6 and GM-CSF mRNA expression and protein

secretion compared to basal in myometrial cells (Fig. 1), decidual cells (Fig. 2) and amnion mesenchymal cells (Fig. 3). In myometrial cells, co-treatment with gallic acid significantly suppressed IL1B- and TNF-induced IL1A, IL6 and GM-CSF mRNA expression and IL6 secretion (Fig. 1A, C, D and E). There was no effect of gallic acid on TNF-induced IL1B mRNA expression or GM-CSF secretion (Fig. 1B and F). In decidual cells, co-treatment with gallic acid significantly suppressed IL1B- and TNF-induced IL1A (Fig. 2A), IL1B (Fig. 2B), IL6 (Fig. 2C) and GM-CSF (Fig. 2E) mRNA expression, and IL6 (Fig. 2D) and GM-CSF secretion (Fig. 2F). In amnion mesenchymal cells, gallic acid significantly suppressed IL1B- and TNF-induced IL6 mRNA expression and secretion (Fig. 3C and D). There was, however, no effect of gallic acid on IL1B- or TNF-induced IL1A, IL1B and GM-CSF mRNA expression or secretion (Fig. 3A, B, E and F).

Effect of gallic acid on IL1B- and TNF-induced proinflammatory chemokines in cells isolated from the myometrium and the foetal membranes

The effect of gallic acid on IL1B- or TNF-induced CCL and CXCL chemokine expression and secretion in primary human myometrial, decidual and amnion mesenchymal cells is depicted in Figs 4, 5 and 6, respectively. For all cells, CCL8 levels in the incubation media were below the sensitivity of the assay.

Treatment with IL1B or TNF significantly increased CCL2, CCL8, CXCL1, CXCL5 and CXCL8 mRNA expression and secretion compared to basal in

Figure 1 Effect of gallic acid on IL1B- and TNF-induced proinflammatory cytokines in human primary myometrial cells. Human primary myometrial cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF with or without 100 μ M gallic acid (GA) for 20 h ($n=6$ patients). (A, B, C and E) IL1A, IL1B, IL6, and GM-CSF mRNA expression were analysed by RT-qPCR. (D and F) The concentrations of IL6 and GM-CSF in the incubation medium were assayed by ELISA. For all graphs, each data point represents an individual patient and the bars represent the mean $\pm$ S.E.M. ^a $P \leq 0.05$ vs IL1B; ^b $P \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

Figure 2 Effect of gallic acid on IL1B- and TNF-induced proinflammatory cytokines in human primary decidual cells. Human primary decidual cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF, with or without 100 μ M gallic acid (GA) for 20 h ($n=6$ patients). (A, B, C and E) IL1A, IL1B, IL6, and GM-CSF mRNA expression were analysed by RT-qPCR. (D and F) The concentrations of IL6 and GM-CSF in the incubation medium were assayed by ELISA. For all graphs, each data point represents an individual patient and the bars represent the mean $\pm$ S.E.M. ^a*P* ≤ 0.05 vs IL1B; ^b*P* ≤ 0.05 vs TNF; repeated measures one-way ANOVA.

myometrial cells (Fig. 4), decidual cells (Fig. 5) and amnion mesenchymal cells (Fig. 6). In myometrial cells, co-treatment with gallic acid significantly suppressed CCL2, CXCL1, CXCL5 and CXCL8 mRNA expression and protein secretion compared to IL1B and TNF treatment (Fig. 4A, B, D, E, F, G, H and I). There was, however, no effect of gallic acid on CCL8 mRNA expression compared with IL1B or TNF (Fig. 4C).

In decidual cells, gallic acid significantly reduced CCL2, CXCL1, CXCL5 and CXCL8 mRNA expression and protein secretion compared to IL1B and TNF treatment (Fig. 5A, B, E, F, G, H and I). There was no effect of gallic acid on CCL8 (Fig. 5C) or CXCL1 (Fig. 5D) mRNA expression compared with IL1B or TNF.

In amnion mesenchymal cells, gallic acid treatment significantly suppressed CXCL8 mRNA expression and

Figure 3 Effect of gallic acid on IL1B- and TNF-induced proinflammatory cytokines in human primary amnion mesenchymal cells. Human primary amnion mesenchymal cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF, with or without 100 μ M gallic acid (GA) for 20 h ($n=6$ patients). (A, B, C and E) IL1A, IL1B, IL6, and GM-CSF mRNA expression were analysed by RT-qPCR. (D and F) The concentrations of IL6 and GM-CSF in the incubation medium were assayed by ELISA. For all graphs, each data point represents an individual patient and the bars represent the mean $\pm$ S.E.M. ^a*P* ≤ 0.05 vs IL1B; ^b*P* ≤ 0.05 vs TNF; repeated measures one-way ANOVA.

Figure 4 Effect of gallic acid on IL1B- and TNF-induced proinflammatory chemokines in human primary myometrial cells. Human primary myometrial cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF, with or without 100 μ M gallic acid (GA) for 20 h ($n=6$ patients). (A, C, D, F and H) CCL2, CCL8, CXCL1, CXCL5 and CXCL8 mRNA expression were analysed by RT-qPCR. (B, E, G and I) The concentrations of CCL2, CXCL1, CXCL5 and CXCL8 in the incubation medium were assayed by ELISA. For all graphs, each data point represents an individual patient and the bars represent the mean $\pm$ S.E.M. ^a $P \leq 0.05$ vs IL1B; ^b $P \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

CCL2, CXCL1, CXCL5 and CXCL8 protein secretion compared with IL1B or TNF (Fig. 6B, E, G, H and I). There was, however, no effect on IL1B- or TNF-induced CCL2, CCL8, CXCL1 and CXCL5 mRNA expression (Fig. 6A, C, D and F).

Effect of gallic acid on MMP expression in human primary decidua and amnion cells

ECM degrading and remodelling enzymes are key mediators involved in the rupture of foetal membranes during parturition. Amnion epithelial cells are major producers of MMP9, where preterm and term labour foetal membranes were found to express higher concentrations of MMP9 than their not-in-labour counterparts (Xu *et al.* 2002). Similar cytokine-induced enzymes, such as MMP1, MMP3 and MMP13, are also thought to act on different cell populations within the foetal membranes, including amnion epithelial, amnion mesenchymal and decidua cells, to facilitate foetal membrane weakening (Weiss *et al.* 2007). Thus, human primary decidua cells and amnion (mesenchymal and epithelial) cells were treated with IL1B or TNF in

the presence or absence of gallic acid to determine the effect of gallic acid on MMP1, MMP3, MMP9 and MMP13 mRNA expression, as well as secreted pro-MMP9 expression.

IL1B or TNF stimulation significantly increased MMP1 and MMP9 mRNA expression, and pro-MMP9 expression compared with basal in decidua (Fig. 7A, C and D), amnion mesenchymal (Fig. 7F, G and H) and amnion epithelial cells (Fig. 7J and K). There was no effect of IL1B or TNF on MMP3 or MMP13 mRNA expression compared with basal in decidua or amnion mesenchymal cells (Fig. 7B, E and I). In decidua cells, gallic acid co-treatment significantly blunted IL1B-induced MMP1 and MMP13 (Fig. 7A and E) mRNA expression, and IL1B- and TNF-induced proMMP9 expression (Fig. 7D). There was no effect of gallic acid on IL1B- or TNF-induced MMP3 and MMP9 mRNA expression (Fig. 7B and C). In amnion mesenchymal cells, gallic acid treatment significantly reduced MMP9 and MMP13 mRNA expression compared with TNF (Fig. 7G and I). However, there was no effect of gallic acid on MMP1 mRNA expression compared with IL1B or TNF (Fig. 7F). There was also no effect of gallic acid on

Figure 5 Effect of gallic acid on IL1B- and TNF-induced proinflammatory chemokines in human primary decidual cells. Human primary decidual cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF, with or without 100 μ M gallic acid (GA) for 20 h ($n=6$ patients). (A, C, D, F and H) CCL2, CCL8, CXCL1, CXCL5 and CXCL8 mRNA expression were analysed by RT-qPCR. (B, E, G and I) The concentrations of CCL2, CXCL1, CXCL5 and CXCL8 in the incubation medium were assayed by ELISA. For all graphs, each data point represents an individual patient and the bars represent the mean $\pm$ S.E.M. ^a $P \leq 0.05$ vs IL1B; ^b $P \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

pro-MMP9 expression compared with IL1B or TNF (Fig. 7H). MMP3 mRNA expression in amnion mesenchymal cells was non-detectable. In epithelial cells, gallic acid co-treatment reduced IL1B-induced proMMP9 expression (Fig. 7K). There was no effect of gallic acid on IL1B- or TNF-induced MMP9 mRNA expression (Fig. 7J). MMP1, MMP3 and MMP13 mRNA expression in amnion epithelial cells were non-detectable.

Effect of gallic acid on COX-2-prostaglandin pathway

Prostaglandin synthase (PTGS2) is the limiting factor in the synthesis of prostaglandin (PG) $F_{2\alpha}$ (Olson 2003). PG $F_{2\alpha}$ then binds with its prostaglandin F receptor (PTGFR) to induce myometrial contractility (Olson 2003) and induce the expression of proinflammatory mediators (Leimert *et al.* 2019). In this study, treatment with IL1B or TNF significantly increased PTGS2 and PTGFR mRNA expression and the release of PG $F_{2\alpha}$ in myometrial cells, compared with basal (Fig. 8A, B and C). The effect of gallic acid was a significant decrease in IL1B-induced PTGS2 mRNA expression and PG $F_{2\alpha}$ release (Fig. 8A and B). Gallic acid also reduced TNF-induced

PG $F_{2\alpha}$ release (Fig. 8B). On the other hand, there was no effect on TNF-induced PTGS2 mRNA expression (Fig. 8A). There was also no effect of gallic acid on either IL1B- or TNF-induced PTGFR mRNA expression (Fig. 8C).

Decidua is also a source of prostaglandins that interacts with myometrial cells to induce uterine activation (Skinner & Challis 1985). Gallic acid co-treatment significantly reduced decidual IL1B-induced PTGS2 mRNA expression and PG $F_{2\alpha}$ release (Fig. 8E and F). There was, however, no effect of gallic acid on TNF-induced PTGS2 mRNA expression and PG $F_{2\alpha}$ release (Fig. 8E and F).

Effect of gallic acid on myometrial cell contractility

Due to the inhibitory effect of gallic acid on the expression of PTGS2 and secretion of uterotonic prostaglandin PG $F_{2\alpha}$ in myometrial cells, we assessed the effect of gallic acid on myometrial cell contractility using a 3D collagen gel assay (Fig. 9). For these studies, human primary myometrial cells were embedded in collagen gels and incubated in TNF with or without gallic acid. TNF treatment significantly reduced collagen

Figure 6 Effect of gallic acid on IL1B- and TNF-induced proinflammatory chemokines in human primary amnion mesenchymal cells. Human primary amnion mesenchymal cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF, with or without 100 μ M gallic acid (GA) for 20 h ($n=6$ patients). (A, C, D, F and H) CCL2, CCL8, CXCL1, CXCL5 and CXCL8 mRNA expression were analysed by RT-qPCR. (B, E, G and I) The concentrations of CCL2, CXCL1, CXCL5 and CXCL8 in the incubation medium were assayed by ELISA. For all graphs, each data point represents an individual patient and the bars represent the mean $\pm$ S.E.M. ^a $P \leq 0.05$ vs IL1B; ^b $P \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

gel area compared with basal by 57.8%, demonstrating increased contraction of the myometrial cells (Fig. 9A). Co-treatment with 100 μ M gallic acid prevented this shrinkage by 49.2% compared with TNF, indicating suppressed myometrial cell contractility. We also assessed the effect of lower concentrations of gallic acid on myometrial contractility and the data are presented in Fig. 9B. Co-treatment with 25 and 50 μ M gallic also significantly reversed TNF-induced shrinkage by 48 and 52.7%, respectively.

Effect of gallic acid on the global protein profile of primary human myometrial and decidual cells

Finally, we sought to identify the potential mechanistic signalling pathways that gallic acid may act through in primary myometrial and decidual cells. Information-dependent acquisition (IDA) and SWATH profile were generated from myometrial and decidual samples using 5 and 6 independent patients, respectively. The IDA library was used to identify peptide ions that were present in SWATH ion profiles. Proteins were identified

and quantified by comparing SWATH-generated peptide ion profiles for each individual sample against the IDA library (PeakView). IDA of mass spectra from all individual samples was initially performed and with a false discovery rate (FRD) of 1% identified 678 and 569 total proteins in decidual and myometrium samples, respectively (Supplementary Tables 1 and 2, see section on [supplementary materials](#) given at the end of this article). The quantification of all proteins identified for each tissue was performed in all individual samples using SWATH MS/MS. The variation in the relative abundance of proteins between controls (i.e. cells treated with TNF only) and treatment (cells co-treated with TNF and gallic acid) was established by comparison with the SWATH profile against the IDA library and presented as volcano plots (Fig. 10A and C).

In myometrial and decidual cells, a total of 47 and 62 statistically significant protein identifications (FDR 1%, and $P < 0.05$) in the relative expression of proteins in treatment compared with control were identified, respectively (Tables 1 and 2). Proteins that were significantly different in gallic acid treated samples

Figure 7 Effect of gallic acid on IL1B- and TNF-induced MMP expression and activity in human primary decidual cells and amnion cells. Human primary decidual (A, B, C, D and E), amnion mesenchymal (F, G, H and I) and amnion epithelial (J and K) cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF, with or without 1100 μ M gallic acid (GA) for 20 h ($n=6$ patients). (A and F) MMP1, (B) MMP3, (C, G and J) MMP9 and (E and I) MMP13 mRNA expression was analysed by qRT-PCR. (D, H and K) ProMMP9 expression in the incubation media was assessed by gelatin zymography. Representative gel image from one patient/tissue is also shown. For all graphs, each data point represents an individual patient and the bars represent the mean $\pm$ S.E.M. ^a $P \leq 0.05$ vs IL1B; ^b $P \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

compared with controls were subjected to ontology and pathway analysis using IPA (Fig. 10B and D).

In the myometrial cells, the significantly different proteins were associated with signalling pathways associated with 'Dermatological Diseases and Conditions, Inflammatory Disease, Organismal Injury and Abnormalities'. Significantly different molecules in these networks include ACTG1, ANXA5, CNN1, COL1A1, COL1A2, COL3A1, COL6A1, COL6A3, DBNL, EEF1B2, MSN, P4HB, PALLD, PDIA3, PDIA6, PDLIM4, SERPINH1, TPM1, TPM2, and TXNRD1. The full list of significantly different protein identifications in myometrial cells is described in Table 1. In the decidual cells, the significantly different proteins were associated with signalling pathways associated with 'Gene Expression, Protein Synthesis, RNA Damage and Repair'. Molecules in these networks include AARS1,

ACO2, ACTR3, ANP32A, DFFA, EIF2S1, HNRNPA2B1, HP1BP3, LETM1, NCL, PA2G4, RAB11A, RAD23B, RPL24, RPL3, RPL30, RPL7A, RPS2, and RPS5. The full list of significantly different protein identifications in decidual cells is described in Table 2.

Discussion

This study is the first to report anti-inflammatory and anti-labour properties of gallic acid in cytokine-stimulated gestational tissues *in vitro*. The data presented in this study found that, in cells isolated from human myometrium and foetal membranes (decidua and amnion mesenchymal cells), gallic acid treatment attenuated TNF-induced mRNA expression and secretion of proinflammatory cytokines and chemokines. In myometrium and decidua, gallic acid treatment attenuated PTGS2 mRNA

Figure 8 Effect of gallic acid on IL1B- and TNF-induced COX-2-prostaglandin pathway proteins in human primary myometrial and decidua cells. Human primary (A, B and C) myometrial and (D and E) decidua cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF, with or without 100 μ M gallic acid (GA) for 20 h ($n=6$ patients). (A and D) PTGS2 and (C) PTGFR mRNA expression and (B and E) PGF_{2 α} concentrations in the incubation media were analysed by qRT-PCR and ELISA, respectively. For all graphs, each data point represents an individual patient and the bars represent the mean $\pm$ S.E.M. ^a $P \leq 0.05$ vs IL1B; ^b $P \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

expression and PGF release and subsequently inhibited collagen gel contraction induced by TNF in myometrial cells. In cells derived from foetal membranes (decidua, amnion mesenchymal and amnion epithelial), gallic acid reduced MMP expression and activity. Using a

global proteomic approach, gallic acid may differentially regulate proteins associated with collagen synthesis, cell contractility and protein synthesis in myometrial and decidua cells.

Pregnancies that culminate in spontaneous preterm birth exhibit abnormal and heightened inflammatory profiles (Wenstrom *et al.* 1998, Kim *et al.* 2013). Often triggered by an initial proinflammatory insult, the activated maternal immune system produces proinflammatory cytokines, such as IL1B and TNF, to allow immune infiltration of the gestational tissues and continued production of proinflammatory cytokines and chemokines to generate a positive feedback loop of inflammation (Lappas *et al.* 2006, Lappas 2017). This proinflammatory cycle then stimulates the production of prostaglandins to activate myometrial contractions (Erkinheimo *et al.* 2000, Rauk & Chiao 2000) as well as increases the expression of foetal membrane weakening enzymes, such as MMP9 (Kumar *et al.* 2006). Using cells derived from human myometrium and foetal membranes (decidua and amnion mesenchyme), we determined the effect of gallic acid on either IL1B or TNF-induced mRNA expression and protein secretion of proinflammatory cytokines and chemokines. We found that gallic acid suppressed the expression and secretion of proinflammatory cytokines and chemokines in all three cell types. IL1B is known to propagate inflammation by stimulating the MyD88 pathway (Lappas 2017), while TNF acts through TRADD signalling (Lim *et al.* 2017b). Interestingly, gallic acid exerted differential effects in response to IL1B- or TNF-induced inflammation and may, therefore, exert stimuli-specific effects. Gallic acid

Figure 9 Effect of gallic acid on TNF-induced human primary myometrial cell contraction. Cell contraction assays were performed using 3D collagen gels embedded with human primary myometrial cells and incubated in 10 ng/mL TNF, with or without (A) 100 μ M gallic acid (GA) ($n=5$ patients) or (B) 25 μ M or 50 μ M GA ($n=3$ patients) for 50 h. The area of the gel was determined using Chemidoc MP. Representative gel contraction image from one patient is also shown. For all graphs, each data point represents an individual patient, and the bars represent the mean $\pm$ S.E.M. ^a $P \leq 0.05$ vs IL1B; ^b $P \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

Figure 10 Effect of gallic acid on global protein profile of primary myometrial and decidual cells. Human primary (A and B) myometrial and (C and D) decidual cells were incubated in 10 ng/mL TNF, with or without 100 μ M gallic acid (GA) for 20 h ($n=5-6$ patients). Proteins were extracted and processed for SWATH and IDA analysis. In each volcano plot (A and C), the horizontal axis represents the fold change and the vertical axis, the P value, where the horizontal dotted line indicates $P=0.05$. Each point represents a protein, with those on the right above the dotted line indicating upregulated proteins and those on the left indicating downregulated proteins. In each signalling network (B and D), the network represents the differentially expressed proteins associated with: (B) Dermatological Diseases and Conditions, Inflammatory Disease, Organismal Injury and Abnormalities, and (D) Gene Expression, Protein Synthesis, RNA Damage and Repair. In each network, the proteins are displayed as nodes and the relationships between the nodes as lines.

may also act in a cell-specific manner; for example, gallic acid significantly suppressed cytokine and chemokine mRNA expression and secretion in myometrial and decidual cells but exerted a weaker effect in amnion mesenchymal cells. Higher doses with longer incubation times of gallic acid treatment may be required to account for these differential effects. Nevertheless, our data

demonstrate that gallic acid exerts suppressive effects on inflammation-induced proinflammatory cytokines and chemokines in myometrial, decidual and amnion mesenchymal cells *in vitro*.

Myometrial activation is mediated through the expression of prostaglandins, such as PGF_{2 α} (Harper & Skarnes 1972, Ruttner *et al.* 2002), and their associated

Table 1 List of proteins significantly upregulated and downregulated by gallic acid treatment in primary human myometrial cells.

ID	Gene symbol	Gene name	Fold change	Expr P-value
Upregulated				
P40925	MDH1	malate dehydrogenase 1	0.596	0.044
P18085	ARF4	ADP ribosylation factor 4	0.442	0.023
P06454	PTMA	prothymosin alpha	0.412	0.047
Q15084	PDIA6	protein disulfide isomerase family A member 6	0.408	0.006
P46782	RPS5	ribosomal protein S5	0.398	0.036
P39656	DDOST	dolichyl-diphosphooligosaccharide--protein glycosyltransferase non-catalytic subunit	0.365	0.020
Q16881	TXNRD1	thioredoxin reductase 1	0.345	0.037
P24534	EEF1B2	eukaryotic translation elongation factor 1 beta 2	0.293	0.007
O00299	CLIC1	chloride intracellular channel 1	0.264	0.041
P29401	TKT	transketolase	0.259	0.006
Q92945	KHSRP	KH-type splicing regulatory protein	0.258	0.048
P08758	ANXA5	annexin A5	0.223	0.045
P14314	PRKCSH	protein kinase C substrate 80K-H	0.207	0.007
Q8WX93	PALLD	palladin, cytoskeletal associated protein	0.178	0.025
P07195	LDHB	lactate dehydrogenase B	0.168	0.020
P07237	P4HB	prolyl 4-hydroxylase subunit beta	0.167	0.004
P10809	HSPD1	heat shock protein family D (Hsp60) member 1	0.104	0.039
P61981	YWHAG	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma	0.102	0.036
P30101	PDIA3	protein disulfide isomerase family A member 3	0.097	0.001
Downregulated				
P02461	COL3A1	collagen type III alpha 1 chain	-2.712	0.034
P08123	COL1A2	collagen type I alpha 2 chain	-1.385	0.011
P02452	COL1A1	collagen type I alpha 1 chain	-1.338	0.003
P51911	CNN1	calponin 1	-0.859	0.050
P50454	SERPINH1	serpin family H member 1	-0.797	0.043
P49411	TUFM	Tu translation elongation factor, mitochondrial	-0.738	0.045
Q9NZN4	EHD2	EH domain containing 2	-0.584	0.049
P07951	TPM2	tropomyosin 2	-0.546	0.013
O43390	HNRNPR	heterogeneous nuclear ribonucleoprotein R	-0.533	0.027
Q9UJU6	DBNL	drebrin like	-0.524	0.039
P12109	COL6A1	collagen type VI alpha 1 chain	-0.463	0.022
P30050	RPL12	ribosomal protein L12	-0.422	0.025
P12111	COL6A3	collagen type VI alpha 3 chain	-0.406	0.042
P35613	BSG	basigin (Ok blood group)	-0.378	0.032
P50479	PDLIM4	PDZ and LIM domain 4	-0.372	0.042
P05556	ITGB1	integrin subunit beta 1	-0.360	0.047
P18669	PGAM1	phosphoglycerate mutase 1	-0.343	0.027
P61254	RPL26	ribosomal protein L26	-0.340	0.018
P17096	HMGA1	high mobility group AT-hook 1	-0.333	0.049
P05976	MYL1	myosin light chain 1	-0.307	0.040
Q03252	LMNB2	lamin B2	-0.264	0.027
Q32P28	P3H1	prolyl 3-hydroxylase 1	-0.258	0.047
P63261	ACTG1	actin gamma 1	-0.246	0.047
P09493	TPM1	tropomyosin 1	-0.238	0.030
P18124	RPL7	ribosomal protein L7	-0.229	0.046
P16401	H1-5	H1.5 linker histone, cluster member	-0.218	0.038
P05787	KRT8	keratin 8	-0.215	0.042
P26038	MSN	moesin	-0.068	0.030

signalling pathway proteins such as PTGS2 (Slater *et al.* 1999) and PTGFR (Senior *et al.* 1993). Animal studies have demonstrated that PGF_{2α} treatment of pregnant mice induces early labour and abortion (Shaw 1983, Sugimoto *et al.* 1997), while PTGFR knockout models delay parturition (Harper & Skarnes 1972). Further to this, elevated expression of these prostaglandin pathway proteins in the myometrium have been positively correlated with spontaneous preterm labour

(Olson 2003, Astle *et al.* 2007, Phillips *et al.* 2011). The decidua is also a source of prostaglandins, with its synthesis increasing during labour (Skinner & Challis 1985). Using human myometrial and decidual cells, we investigated the effect of gallic acid on inflammation-induced expression of contraction-associated proteins and prostaglandins. Gallic acid reduced inflammation-induced PTGS2 mRNA expression and secretion of PGF_{2α}. In myometrial and decidual cells, gallic acid

Table 2 List of proteins significantly upregulated and downregulated by gallic acid treatment in primary human decidual cells.

ID	Gene symbol	Gene name	Fold change	Expr P-value
Upregulated				
P16949	STMN1	stathmin 1	0.842	0.024
P07910	HNRNPC	heterogeneous nuclear ribonucleoprotein C	0.720	0.048
Q9UBS4	DNAJB11	DnaJ heat shock protein family (Hsp40) member B11	0.619	0.022
P07305	H1-0	H1.0 linker histone	0.550	0.007
Q99729	HNRNPAB	heterogeneous nuclear ribonucleoprotein A/B	0.509	0.014
P62491	RAB11A	RAB11A, member RAS oncogene family	0.450	0.002
P05106	ITGB3	integrin subunit beta 3	0.385	0.006
Q14624	ITIH4	inter-alpha-trypsin inhibitor heavy chain 4	0.379	0.023
P09429	HMGB1	high mobility group box 1	0.378	0.009
O00273	DFFA	DNA fragmentation factor subunit alpha	0.375	0.024
P13647	KRT5	keratin 5	0.369	0.010
Q99798	ACO2	aconitase 2	0.367	0.012
Q9UHB6	LIMA1	LIM domain and actin binding 1	0.365	0.022
Q99733	NAP1L4	nucleosome assembly protein 1 like 4	0.357	0.002
Q14103	HNRNPD	heterogeneous nuclear ribonucleoprotein D	0.343	0.023
Q14008	CKAP5	cytoskeleton associated protein 5	0.322	0.013
P22626	HNRNPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1	0.292	0.048
P01889	HLA-B	major histocompatibility complex, class I, B	0.285	0.015
P11413	G6PD	glucose-6-phosphate dehydrogenase	0.284	0.026
Q92896	GLG1	golgi glycoprotein 1	0.268	0.023
Q9UQ80	PA2G4	proliferation-associated 2G4	0.255	0.016
P38117	ETFB	electron transfer flavoprotein subunit beta	0.252	0.048
P39023	RPL3	ribosomal protein L3	0.240	0.027
Q5SSJ5	HP1BP3	heterochromatin protein 1 binding protein 3	0.228	0.019
P62888	RPL30	ribosomal protein L30	0.225	0.042
P46782	RPS5	ribosomal protein S5	0.225	0.049
P54727	RAD23B	RAD23 homolog B, nucleotide excision repair protein	0.222	0.044
Q92973	TNPO1	transportin 1	0.209	0.034
P49327	FASN	fatty acid synthase	0.207	0.019
P39687	ANP32A	acidic nuclear phosphoprotein 32 family member A	0.206	0.027
O95782	AP2A1	adaptor related protein complex 2 subunit alpha 1	0.195	0.010
P19338	NCL	nucleolin	0.191	0.037
P49411	TUFM	Tu translation elongation factor, mitochondrial	0.189	0.035
P05198	EIF2S1	eukaryotic translation initiation factor 2 subunit alpha	0.185	0.025
P02545	LMNA	lamin A/C	0.181	0.021
P62424	RPL7A	ribosomal protein L7a	0.176	0.011
P0DME0	SETSIIP	SET like protein	0.160	0.017
Q15293	RCN1	reticulocalbin 1	0.158	0.023
P13798	APEH	acylaminoacyl-peptide hydrolase	0.144	0.005
P52907	CAPZA1	capping actin protein of muscle Z-line subunit alpha 1	0.138	0.047
P61158	ACTR3	actin related protein 3	0.127	0.026
P23588	EIF4B	eukaryotic translation initiation factor 4B	0.125	0.022
P15880	RPS2	ribosomal protein S2	0.103	0.044
Q99426	TBCB	tubulin folding cofactor B	0.097	0.045
Q8NC51	SERBP1	SERPINE1 mRNA binding protein 1	0.080	0.039
Downregulated				
P02452	COL1A1	collagen type I alpha 1 chain	-1.248	0.007
P05120	SERPINB2	serpin family B member 2	-0.909	0.013
Q02790	FKBP4	FKBP prolyl isomerase 4	-0.876	0.022
Q86Y46	KRT73	keratin 73	-0.852	0.007
P08123	COL1A2	collagen type I alpha 2 chain	-0.841	0.006
P02461	COL3A1	collagen type III alpha 1 chain	-0.743	0.009
P12109	COL6A1	collagen type VI alpha 1 chain	-0.637	0.011
P49588	AARS1	alanyl-tRNA synthetase 1	-0.306	0.048
P05362	ICAM1	intercellular adhesion molecule 1	-0.276	0.008
Q01813	PFKP	phosphofructokinase, platelet	-0.275	0.008
O15460	P4HA2	prolyl 4-hydroxylase subunit alpha 2	-0.261	0.006
P83731	RPL24	ribosomal protein L24	-0.210	0.014
Q8N1G4	LRRC47	leucine rich repeat containing 47	-0.204	0.047
Q96AY3	FKBP10	FKBP prolyl isomerase 10	-0.203	0.026
O95202	LETM1	leucine zipper and EF-hand containing transmembrane protein 1	-0.194	0.028
P04406	GAPDH	glyceraldehyde-3-phosphate dehydrogenase	-0.179	0.023
Q9Y230	RUVBL2	RuvB like AAA ATPase 2	-0.133	0.009

exerted differential effects in response to TNF and IL1B; in both cell types, gallic acid reduced IL1B-induced PTGS2 mRNA expression and PGF_{2α} secretion, but only in myometrial cells did gallic acid suppress TNF-induced PGF_{2α} secretion. It is possible that different doses and incubation times are also required to affect distinct stages of prostaglandin signalling.

We next investigated the effect of gallic acid on myometrial cell contractility through the use of a collagen gel assay embedded with primary human myometrial cells. Gallic acid treatment was found to block TNF-induced shrinkage of the collagen gel, suggesting gallic acid was able to prevent myometrial cell contractility. A previous study found gallic acid was able to suppress F-actin formation and myosin light chain phosphorylation in TGF-β1-stimulated collagen gel contraction (Hsieh *et al.* 2016). Together, these data suggest gallic acid likely may act directly on downstream mediators involved in cell contraction mediated by TNF. That gallic acid can mitigate inflammation-induced prostaglandin synthesis in both myometrial and decidual cells, as well as suppress myometrial cell contractility, suggests that it may be a potential tocolytic for the prevention of preterm labour.

Preterm premature rupture of membranes (pPROM) occurs through the weakening of the foetal membranes (Lannon *et al.* 2014). Proinflammatory cytokines are known to regulate the expression and activity of MMPs for the breakdown and digestion of the extracellular matrix (Kumar *et al.* 2006). To determine the effect of gallic acid on the MMPs, we stimulated cells derived from the foetal membranes (decidua, amnion mesenchyme and amnion epithelium) with IL1B and TNF. We found that gallic acid differentially regulated mRNA expression of MMP1, MMP9 and MMP13 in the three cell types. Furthermore, gallic acid inhibited expression of pro-MMP9 in the decidua and amnion epithelium. MMP1, MMP9 and MMP13 are present in amniotic fluid and implicated to play a role in foetal membrane weakening (Athayde *et al.* 1998, Maymon *et al.* 2000). In particular, MMP9 protein expression is demonstrated to increase in foetal membranes during and after labour, compared with before labour (Tsatas *et al.* 1999). The finding that gallic acid can regulate MMP mRNA expression, and especially reduce MMP9 expression, in cells derived from the foetal membranes suggest that gallic acid may be able to prevent foetal membrane weakening associated with pPROM.

A global proteomic approach was used to identify the mechanistic pathways through which gallic acid may be acting in myometrial and decidual cells. Interestingly, for both myometrial and decidual cells, gallic acid was found to alter the regulation of proteins involved in collagen synthesis and cellular contractility, as well as protein synthesis and nuclear integrity. Notably, in myometrial cells, gallic acid co-treatment was found to downregulate proteins associated with cell contractility

and movement. These include myosin light chain 1 and tropomyosins 1 and 2, all of which are thought to directly bind with actin to induce cellular contractility (Marston & El-Mezgueldi 2008, Nguyen-Ngo & Lappas 2020). Gallic acid was also found to significantly downregulate collagen proteins, including type 1, type 3 and type 6 collagens in both myometrial and decidual cells. Fibrillar collagens type I and III are necessary strength-providing components of the extracellular matrix (ECM) (Kisalus *et al.* 1987, Malak *et al.* 1993, Mylona *et al.* 1995), where extensive remodelling of the myometrium and the foetal membranes leads to increasing concentration with advancing gestation (Puistola *et al.* 1993). In particular, ECM proteins, including collagens, have been found to increase in labouring rat myometrium to induce myometrial contractions (Shynlova *et al.* 2004, 2009) as well as in human preterm premature rupture of membranes (Meirowitz *et al.* 2002) and preterm labour (Shankar *et al.* 2010). Together, these findings suggest that gallic acid may influence tissue remodelling in both myometrium and foetal membranes. In conjunction with our functional collagen gel contractility data, gallic acid may also directly target downstream mechanisms to reduce TNF-induced myometrial cell contractility in collagen gels.

Gallic acid also downregulated the adhesion molecule ICAM1, which is known to play a role in leukocyte infiltration of the myometrium and foetal membranes during labour (Thomson *et al.* 1999, Ledingham *et al.* 2001). Supporting this, our study found that gallic acid treatment significantly reduced soluble ICAM1 secretion in the incubation media of TNF-stimulated decidual cells (Supplementary Fig. 1). Together, these data suggest that gallic acid may limit leukocyte infiltration into the decidua to block the production of proinflammatory cytokines and chemokines and ultimately prevent the proinflammatory cycle associated with preterm birth.

Other changes induced by gallic acid treatment include differential expression of proteins involved in protein synthesis and nuclear integrity in both myometrial and decidual cells.

In myometrial cells, proteins associated with chromatin and nuclear structure, and ribosomal components were downregulated, while proteins involved in protein translation and posttranslational modifications were upregulated. In decidual cells, proteins associated with eukaryotic translation factors, ribosomal components and posttranslational modifications were upregulated. We have previously demonstrated dysregulated expression of molecules involved in protein synthesis in myometrium and foetal membranes from labouring women compared with non-labouring women, which was associated with the production of proinflammatory and pro-labour mediators (Liong & Lappas 2018). Similarly, preterm infected or ruptured membranes also exhibited dysregulated expression of protein synthesis molecules compared with non-infected or

intact membranes (Liong & Lappas 2018). These data suggest that gallic acid may be able to reverse labour-induced changes in protein synthesis machinery to reduce the inflammatory response in the myometrium and foetal membranes. Notably, in decidual cells, gallic acid upregulated eukaryotic translation initiation factor (EIF)4B, which acts as a cofactor for EIF4 subunit A (Sen *et al.* 2016). Aside from regulating protein synthesis, EIF4 is also associated with cell cycle progression and apoptosis resistance (Ruggero *et al.* 2004, Wendel *et al.* 2004, Holcik & Sonenberg 2005, Proud 2015, Zhan *et al.* 2015). Gallic acid was also found to upregulate proteins associated with nucleic acid structure and integrity, as well as nuclear membrane structure and assembly in decidual cells, indicating that gallic acid may exert a protective effect against inflammation-induced damage of nucleic material. Altogether, this may suggest that gallic acid programs cells for survival in proinflammatory environments, which holds important implications for the foetal membranes, where apoptosis and cell death are a mechanism for the rupture of the membranes (Fortunato *et al.* 2000, Reti *et al.* 2007). These findings suggest that gallic acid may act on a number of signalling targets to prevent myometrial contractions and foetal membrane rupture associated with preterm birth.

Cervical ripening and softening is the third component of the common pathway of parturition and involves MMP-induced remodelling and degradation to allow the passage of the fetus through the cervical canal (Vink & Myers 2018). Inflammation is also thought to contribute to this process in both preterm and term labouring women (Dobyns *et al.* 2015, Dubicke *et al.* 2016, Yellon 2017). Whether gallic acid can also prevent inflammation associated with cervical ripening is an avenue of further research.

This study is not without limitations. Spontaneous preterm birth is a complex disease where other infectious agents, such as bacterial FSL-1 or viral poly(I:C), may also contribute to the pathophysiology of preterm birth by triggering distinct pathways to the proinflammatory cytokines (Lim *et al.* 2017a). Thus, the findings presented here should be interpreted with some caution. Despite this, the study's strengths lie in the use of four distinct primary human cell lines derived from the critical tissues involved in preterm birth, including the myometrium and the foetal membranes (decidua, amnion mesenchyme and amnion epithelium). In addition, the effects of gallic acid were measured in response to either IL1B or TNF, each of which induces inflammation through distinct pathways.

The findings in this study warrant future preclinical studies on the safety and efficacy of gallic acid in pregnant animal models. Previous non-pregnant human clinical trials, as well as a pregnant rat study, have found safety in gallic acid supplementation (Booth *et al.* 2010, Fairus *et al.* 2018, Ferk *et al.* 2018). Pregnant rats treated

with up to 860 mg/kg gallic acid (equivalent to 6.8 g/mL) from gd 6 to 20 were deemed healthy, with no adverse effect on maternal or foetal outcomes (Booth *et al.* 2010). Of note, non-pregnant human clinical trials found that daily supplementation of 450 mg gallic acid equivalent for 60 days was safe for consumption (Fairus *et al.* 2018). By comparison, our study used a dose of 100 µM or 0.17 mg/mL gallic acid. It is imperative that future preclinical studies identify the safety and efficacy of gallic acid in preventing spontaneous preterm birth using in vivo mouse studies.

Despite impacting 15 million pregnancies every year, current prevention therapeutics for spontaneous preterm birth possess limited effectiveness in some, but not all, subsets of at-risk pregnancies (Beck *et al.* 2010, Haas *et al.* 2012). In this study, we assessed the effect of the polyphenol gallic acid on cytokine-stimulated gestational tissues in vitro. We found that gallic acid treatment was able to suppress the production of key mediators involved in myometrial activation and the rupture of foetal membranes, including proinflammatory cytokines, chemokines, prostaglandin pathway-associated proteins, uterotonics and MMPs. Global proteomic data suggest that gallic acid may differentially regulate proteins associated with collagen synthesis, cell contractility and gene expression in myometrial and decidual cells. Altogether, these data suggest that gallic acid may be a novel therapeutic to prevent myometrial contractions and rupture of membranes associated with spontaneous preterm birth. Additional studies are warranted to determine if gallic acid may be able to prevent preterm birth and improve foetal outcome in an in vivo animal model.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0249>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M L designed the study and critically reviewed the manuscript. C N-N conducted the experiments, performed data analysis and wrote the initial draft of the manuscript. C S performed mass spectrometry analysis. A L provided technical assistance. J C W critically reviewed the study and the manuscript.

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