American Journal of Immunology

Original Research Paper

Punicalagin Suppresses Mediators Involved in Labor Onset and Progression *in vitro*

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Article history
Received: 17-03-2021
Revised: 04-07-2021
Accepted: 22-08-2021

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Abstract: Spontaneous preterm birth is the leading cause of neonatal morbidity and mortality. Inflammation plays a central role in the activation of myometrial contractions and rupture of fetal membranes associated with spontaneous preterm birth. The pomegranate polyphenol punicalagin is known to possess anti-inflammatory properties. In this study, we aimed to determine the effect of punicalagin on pro-inflammatory and pro-labor mediators in an *in vitro* model of intrauterine inflammation associated with preterm birth. Primary human cells isolated from myometrium, decidua and fetal membranes (amnion mesenchyme) were stimulated with or without IL1B or TNF, in the absence or presence of punicalagin. Punicalagin blocked inflammation-induced expression of pro-inflammatory cytokines, chemokines and extracellular matrix degrading enzymes in primary myometrial, decidual and amnion cells. Punicalagin also suppressed inflammation-induced myometrial activation as evidenced by a decrease in PTGS2 mRNA expression, $PGF_{2\alpha}$ secretion and myometrial cell contractility *in situ*. Overall, punicalagin may block pro-inflammatory and pro-labor mediators in human gestational tissues. Further study should identify if punicalagin can delay LPS-induced spontaneous preterm birth in an *in vivo* mouse model.

Keywords: Punicalagin, Preterm Birth, Myometrial Activation, Membrane Rupture, Inflammation

Introduction

Spontaneous preterm birth is the leading cause of neonatal morbidity and mortality and affects up to 20% of all pregnancies (Beck *et al*., 2010). Survivors of preterm birth face lifelong neurological disabilities, including cerebral palsy, developmental delay and hearing and visual impairment (Mwaniki *et al*., 2012). In addition to these significant health complications, spontaneous preterm birth creates heavy economic burden on the healthcare system - preterm birth leads to initial hospitalisation costs of up to US$ 576 972 per baby born at 24 weeks and continued long-term public sector costs of US$39 329 per preterm survivor reaching 18 years (Petrou *et al*., 2019).

Inflammation is a known trigger for more than half of all spontaneous preterm births (75%) (Goldenberg *et al*., 2000, Romero *et al*., 1988, Gilman-Sachs *et al*., 2018). Infectious or sterile pro-inflammatory insults activate the maternal immune system to induce a pro-inflammatory cascade of events, including leukocyte infiltration into the gestational tissues (Thomson *et al*., 1999, Hamilton *et al*., 2012) and upregulation of pro-inflammatory cytokines (IL1A, IL1B, IL6, GM-CSF, TNF) and Chemokines (CCL and CXCL chemokines). These cytokines induce further production of pro-inflammatory cytokines and chemokines (Lappas, 2017, Lappas *et al*., 2006, Lim *et al*., 2017) and other pro-labor mediators (Kumar *et al*., 2006, Rauk and Chiao, 2000, Chen *et al*., 2020). These include the contraction-associated proteins prostaglandin-endoperoxide synthase 2 (PTGS2) and the uterotonic prostaglandin F2α (PGF2α) to stimulate myometrial contractility and Extra cellular Matrix (ECM) degrading and remodelling enzymes to induce weakening and rupture of the fetal membranes (Vadillo-Ortega and Estrada-Gutierrez, 2005, Olson and Ammann, 2007).

Current therapeutics to delay preterm birth are beneficial to only a specific subset of at-risk women: Progesterone is only effective in women presenting with a short cervix, while tocolytic therapies delay preterm birth for only 48 h (Haas *et al*., 2012). These modest effects are likely due to little impact of the therapeutics on the inflammatory response that drives spontaneous preterm birth. Therefore, it is essential to identify new anti-inflammatory interventions that may reduce intrauterine inflammation and ultimately prevent preterm birth and its associated lifelong consequences.

Recent evidence suggests that plant-based diets, rich in polyphenols, are associated with reduced incidence of preterm birth (Englund-Ögge *et al*., 2014, Myhre *et al*., 2013). Polyphenols are natural, plant-based compounds that have demonstrated anti-inflammatory properties (Manach *et al*., 2004). We have previously shown that three classes of polyphenols (flavonoids: Nobiletin, naringenin, apigenin, silibinin, luteolin and kaempferol; lignans: Honokiol; and phenolic acids: Curcumin, gallic acid) can suppress inflammation-induced expression of pro-inflammatory and pro-labor mediators in placenta, myometrium and fetal membranes (Wijesuriya and Lappas, 2018, Morwood and Lappas, 2014, Lim *et al*., 2014, Lim *et al*., 2013c, Wall *et al*., 2013, Nguyen-Ngo *et al*., 2020). Punicalagin is an ellagitannin, a type of phenolic acid, commonly found in pomegranates, East Indian almonds and the medicinal velvet bushwillow (Rothwell *et al*., 2013, Asres *et al*., 2001, Marzouk *et al*., 2002). Several studies report anti-inflammatory properties of punicalagin *in vitro* and *in vivo* (Bensaad *et al*., 2017, Xu *et al*., 2014, Kang *et al*., 2019, Peng *et al*., 2015). In murine macrophages, punicalagin inhibited LPS-induced PTGS2 mRNA expression and prostaglandin release, as well as pro-inflammatory cytokine (IL1B, IL6 and TNF) mRNA expression and release (Bensaad *et al*., 2017, Xu *et al*., 2014). In high-fat-diet obese mice, punicalagin inhibited cytokine (IL1B, IL6 and TNF) and Chemokine (CCL2) mRNA expression in white adipose tissue (Kang *et al*., 2019), while in an animal model of LPS-induced lung injury, punicalagin inhibited leukocyte infiltration and reduced cytokine (IL1B, IL6 and TNF) levels in lung tissue (Peng *et al*., 2015). However, there are no studies investigating the effect of punicalagin on either *in vitro* or *in vivo* models of spontaneous preterm birth.

The aim of this study was to investigate the effect of punicalagin on the expression of mediators involved in the rupture of fetal membranes and myometrial activation. IL1B and TNF are key cytokines known to induce prostaglandin pathway proteins and ECM-degrading enzymes in myometrium and fetal membranes (Lappas, 2017, Lim *et al*., 2017). Thus, primary human cells were isolated from pregnant non-laboring myometrium, decidua and fetal membranes (amnion mesenchymal) and incubated with IL1B or TNF to generate an *in vitro* model of inflammation-induced preterm birth. Myometrial cells were also used in collagen gel assays to examine the effect of punicalagin on myometrial cell contractility.

Methods

Human Tissue Collection

Mercy Hospital for Women's Research and Ethics Committee granted ethics approval (Mercy Health, Ethics approval number R04-29) for tissue collection. All participating women provided written informed consent. All experiments were then performed in accordance with relevant guidelines and regulations. Myometrium was excised from the lower uterine segment and placenta collected together with attached fetal membranes. Tissues were obtained from healthy pregnant women (body mass index <30 kg/m^2) undergoing elective Caesarean section in the absence of labor to deliver a single healthy infant at term (37-40 weeks gestation). Women were excluded if they were smokers, or had vascular/renal complications, asthma, multiple gestations, acute fetal distress, preeclampsia, chorioamnionitis, placental abruption, or any other underlying medical conditions. All tissues were delivered to the laboratory and processed within 15 min of delivery.

Primary Cell Culture

Myometrial cells (Lim *et al*., 2013a), decidual cells (Araujo *et al*., 2018) and mesenchymal cells (Lim and Lappas, 2019) were isolated and cultured as previously described. Decidual tissue was collected from the chorion layer of fetal membranes. Cells were grown in DMEM/F-12 containing 1% penicillin-streptomycin and 10% heat-inactivated bovine serum at 37°C in a humidified incubator of 21% O_2 (myometrial cells) or 8% O_2 (decidual and amnion mesenchymal cells). Once confluent, cells were split into 48-well plates and treated with 1 ng/mL IL1B or 10 ng/mL TNF, with or without 10 μM punicalagin. The optimised dose of punicalagin was based on previous literature (Zhong *et al*., 2015, Chen *et al*., 2012) and a dose response test (data not shown). After 20 h incubation, cells and media were collected separately and stored at -80°C until analysis by ELISA, real-time quantitative Polymerase Chain Reaction (RT-qPCR) or gelatin zymography as detailed below. Each experiment was performed on samples obtained from 5 independent patients.

Myometrial Collagen Gel Contractility Assay

Cell contraction assays were performed as previously described (Wijesuriya and Lappas, 2018). Primary human myometrial cells were re-suspended in 0.25 mL DMEM/F12 (containing 10% FBS) 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 in 37°C to allow polymerization (approx. 30 min) and then treated with 10 ng/mL TNF in the absence or presence of 10 μM punicalagin. 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 software

(Bio-Rad Laboratories, Hercules, CA, USA). Experiments were performed from myometrium obtained from 5 patients.

Enzyme-Linked Immunosorbent Assay

Media from the tissue explants were assayed using a sandwich ELISA to determine the protein concentrations of IL6, CXCL8 and GM-CSF (Life Technologies; Mulgrave, Vic, Australia) and IL1A, IL1B, CCL2, CXCL1 and CXCL5 (RnD Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The release of $PGF_{2\alpha}$ into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's instructions (Cayman Chemical Company; Ann Arbor, MI, USA). The calculated interassay and intraassay Coefficients of Variation (CV) were all less than 10%. The limit of detection (defined as 2 SD from the zero standard) were as follows: IL1A, 7.8; IL1B, 3.9; IL6, 15.6; GM-CSF, 6; CCL2, 15.6; CXCL1, 31.3; CXCL5, 15.6; and CXCL8, 12.5 pg/mL.

RNA Extraction and RT-qPCR

Total RNA was extracted from tissues using TRI Reagent (Sigma-Aldrich, Saint Louis, MO, USA) and converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) as previously described (Wijesuriya and 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; Chadstone Centre, Vic, Australia) were used to perform RT-PCR on the CFX384 Real-Time PCR detection system (Bio-Rad Laboratories, Gladesville, NSW, Australia). Average gene Ct values for the *in vitro* studies were normalised against the housekeeping genes tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein Zeta (YWHAZ) and succinate dehydrogenase complex Subunit A (SDHA). Experimental treatment had no effect on the mRNA expression of SDHA or YWHAZ. The comparative Ct method was used to determine fold differences.

Gelatin Zymography

Gelatin zymography was used to assess the expression of pro MMP9 in incubation media 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; Gladesville, NSW, Australia) and inverted and densitometry was performed using Quantity One image analysis software (Bio-Rad Laboratories; Gladesville, NSW, Australia).

Statistical Analysis

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Shapiro-Wilk test was used to test the normality of all the data. Data for Fig. 1-7 were analysed by a repeated measures one-way ANOVA (with LSD post-hoc testing to discriminate among the means); non-normally distributed data were logarithmically transformed before analysis. Statistical significance was ascribed to a P value ≤ 0.05. Data were expressed as mean ± Standard Error of the Mean (SEM).

Results

Effect of Punicalagin on Pro-Inflammatory Cytokines in Primary Myometrial, Decidual and Amnion Cells

The effect of punicalagin on mRNA expression and protein secretion of pro-inflammatory cytokines in primary myometrial, decidual and amnion cells is demonstrated in Fig. 1. For all cells, the concentrations of IL1A, IL1B and GM-CSF in the incubation media were below the sensitivity of the assay.

In primary human myometrial, IL1B and TNF significantly upregulated IL1A, IL1B, IL6 and GM-CSF mRNA expression and IL6 protein concentration compared with basal (Fig. 1A-E). Punicalagin significantly suppressed IL1B-induced IL1A, IL6 and GM-CSF mRNA expression and IL6 protein concentration (Fig. 1A, C-E) and TNF-induced IL1A mRNA expression (Fig. 1A) and IL6 protein concentration (Fig. 1D). However, there was no effect of punicalagin on TNF-induced IL1B, IL6 or GM-CSF mRNA expression (Fig. 1B, C, E).

In primary human decidual cells, IL1B significantly increased IL1A, IL6 and GM-CSF mRNA expression (Fig. 1F, H, J) compared to basal and TNF increased IL1A, IL1B, IL6 and GM-CSF mRNA expression (Fig. 1F-H, J) compared with basal. There was no effect of IL1B or TNF on IL6 release compared with basal (Fig. 1I). Punicalagin significantly reduced IL1B-induced IL6 protein secretion and TNF-induced IL1A and IL1B mRNA expression and IL6 release (Fig. 1F, G, I). There was, however, no effect of punicalagin on IL1B-induced IL1A, IL6 or GM-CSF mRNA expression (Fig. 1F, H, J) or TNF-induced IL6 or GM-CSF mRNA expression (Fig. 1H, J).

In primary human amnion mesenchymal cells, IL1B significantly induced IL6 mRNA expression and release (Fig. 1N, O), while TNF significantly increased IL1A, IL1B and IL6 mRNA expression and secretion (Fig. 1K-O) compared with basal. There was no effect of IL1B on IL1A or GM-CSF mRNA expression (Fig. 1K, P) or TNF on GM-CSF mRNA expression (Fig. 1P). Punicalagin significantly downregulated TNF-induced IL1A and IL1B mRNA expression (Fig. 1K, L). There was no effect of punicalagin on IL1B-induced IL1A, IL6 or GM-CSF mRNA expression and IL6 secretion (Fig. 1K, N-P). There was also no effect of punicalagin on TNF-induced IL6 or GM-CSF mRNA expression and IL6 secretion (Fig. 1N-P).

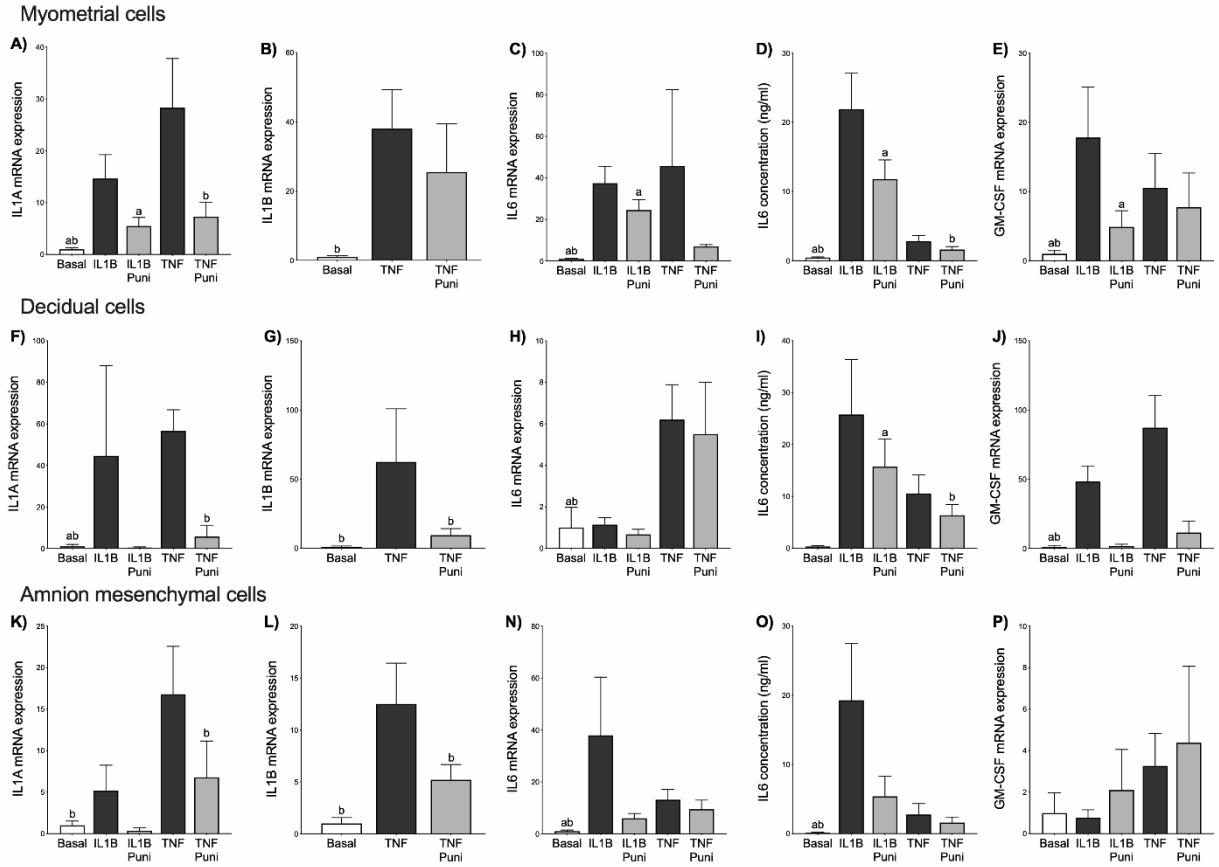

Fig. 1: Effect of punicalagin on pro-inflammatory cytokine expression and secretion in primary myometrial, decidual and amnion cells. Human primary (A-E) myometrial, (F-J) decidual and (K-P) amnion cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF with or without 10 mm punicalagin (Puni) for 20 h (n = 5 patients). (A, F, K) IL1A, (B, G, L) IL1B, (C, H, N) IL6, and (E, J, P) GM-CSF mRNA expression were analysed by RT-qPCR. (D, I, O) IL6 concentration in the incubation medium was assayed by ELISA. All data is displayed as mean ± SEM. Statistical significance was determined using repeated measures one-way ANOVA; the symbol 'a' denotes statistical significance compared with IL1B ($P \leq 0.05$), while the symbol 'b' denotes statistical significance compared with TNF ($P \leq 0.05$)

Effect of Punicalagin on Pro-Inflammatory Chemokines in Primary Myometrial, Decidual and Amnion Cells

Next, we examined the effect of punicalagin on pro-inflammatory CCL and CXCL chemokines in primary myometrial cells (Fig. 2), decidual cells (Fig. 3) and amnion mesenchymal cells (Fig. 4). In primary human myometrial cells, IL1B and TNF significantly upregulated CCL2, CCL8, CXCL1, CXCL5 and CXCL8 mRNA expression and secretion compared with basal (Fig. 2A-I). Punicalagin significantly downregulated IL1B- and TNF-induced CXCL8 mRNA expression (Fig. 2H); however there was no effect of punicalagin on IL1B- and TNF-induced CCL2, CCL8, CXCL1 or CXCL5 mRNA expression (Fig 2A, C, D, F). Punicalagin significantly reduced IL1B- and TNF-induced protein secretion of CCL2, CXCL1, CXCL5 and CXCL8 (Fig 2B, E, G, I).

In primary human decidual cells, IL1B significantly increased CCL2, CXCL1, CXCL5 and CXCL8 mRNA expression and protein secretion (Fig. 3A,B,D-I). TNF significantly induced CCL2, CXCL1, CXCL5 and CXCL8 mRNA expression and CCL2, CXCL1 and CXCL8 protein concentration compared with basal (Fig. 3A, B, D-F, H, I). There was no effect of IL1B or TNF on CCL8 mRNA expression (Fig. 3C). There was also no effect of TNF on CXCL5 secretion (Fig. 3G). Punicalagin significantly reduced IL1B- and TNF-induced CXCL8 mRNA expression and CCL2, CXCL1, CXCL5 and CXCL8 protein release (Fig. 3B, E, G-I). There was no effect of punicalagin on CCL2, CCL8, CXCL1 or CXCL5 mRNA expression in the presence of IL1B or TNF (Fig. 3A, C, D, F).

In primary human amnion mesenchymal cells, IL1B and TNF upregulated CCL2, CXCL1, CXCL5 and CXCL8 mRNA expression and CXCL5 and CXCL8 protein

secretion (Fig. 4A, D, F-I) compared with basal. IL1B also induced CXCL1 protein secretion (Fig. 4E); however, there was no effect of IL1B or TNF on CCL2 protein release (Fig. 4B) or CCL8 mRNA expression (Fig. 4C). There was also no effect of TNF on CXCL1 protein release (Fig. 4E). Punicalagin treatment significantly suppressed IL1B-induced CCL8 and CXCL8 mRNA expression and CXCL1, CXCL5, CXCL8 protein secretion (Fig. 4C, E, G-I). Punicalagin treatment also significantly reduced TNF-induced CXCL1, CXCL5 and CXCL8 mRNA expression (Fig. 4D, F, H) and CCL2 and CXCL8 protein release (Fig. 4B, I).

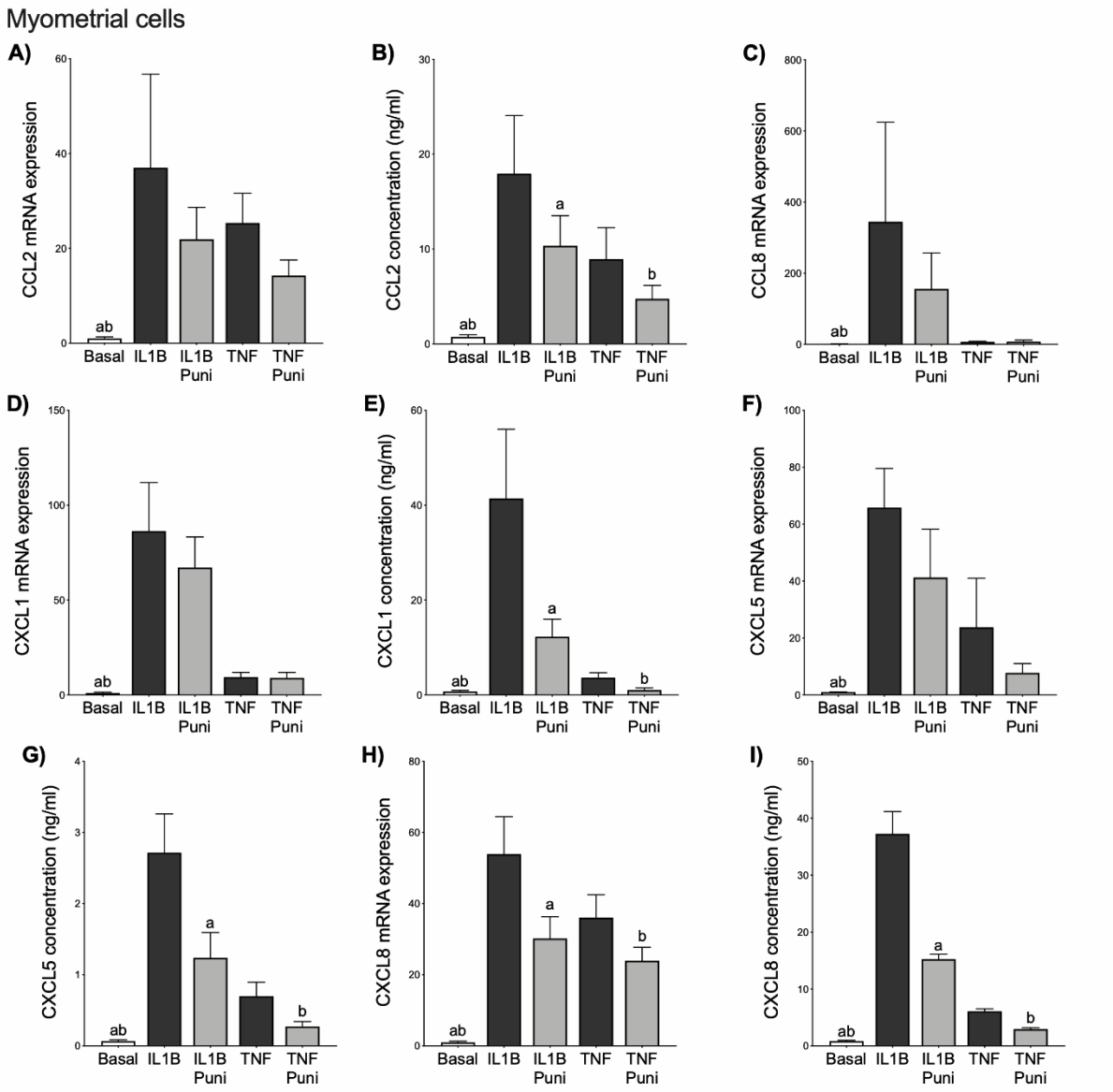

Fig. 2: Effect of punicalagin on pro-inflammatory chemokine expression and secretion in primary myometrial cells. Human primary myometrial cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF with or without 10 µm punicalagin (Puni) for 20 h (n = 5 patients). (A,C,D,F,H) CCL2, CCL8, CXCL1, CXCL5 and CXCL8 mRNA expression were analysed by RT-qPCR. (B,E,G,I) CCL2, CXCL1, CXCL5 and CXCL8 concentrations in the incubation medium were assayed by ELISA. All data is displayed as mean ± SEM. Statistical significance was determined using repeated measures one-way ANOVA; the symbol 'a' denotes statistical significance compared with IL1B ($P \leq 0.05$), while the symbol 'b' denotes statistical significance compared with TNF ($P \leq 0.05$)

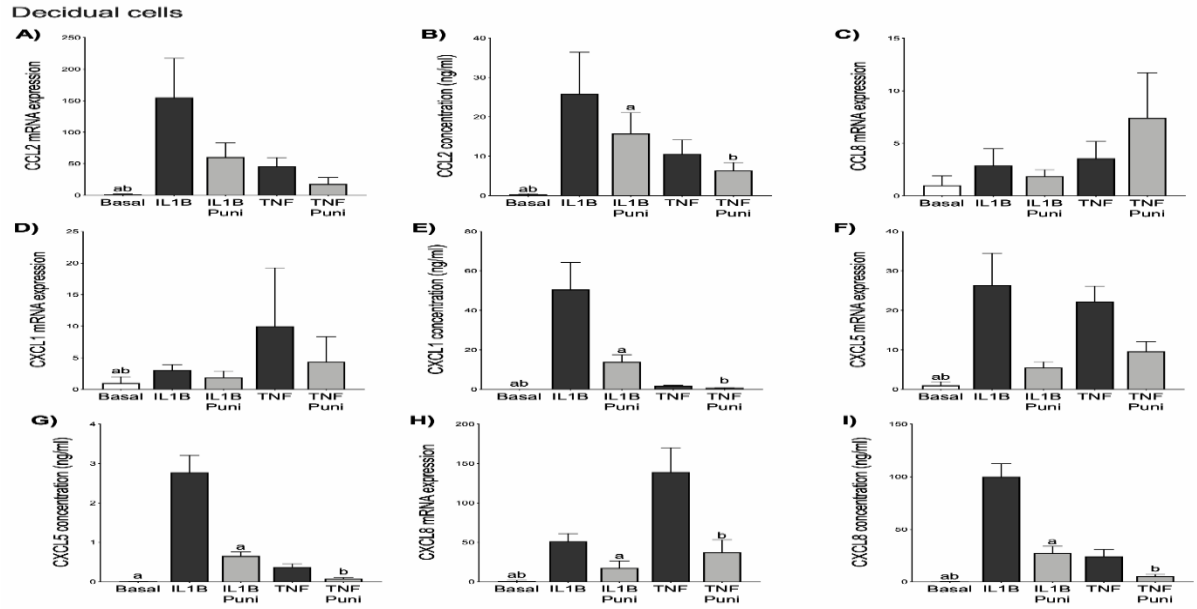

Fig. 3: Effect of punicalagin on pro-inflammatory chemokine expression and secretion in primary decidual cells. Human primary decidual cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF with or without 10 μm punicalagin (Puni) for 20 h (n = 5 patients). **(A,C,D,F,H)** CCL2, CCL8, CXCL1, CXCL5 and CXCL8 mRNA expression were analysed by RT-qPCR. **(B,E,G,I)** CCL2, CXCL1, CXCL5 and CXCL8 concentrations in the incubation medium were assayed by ELISA. All data is displayed as mean ± SEM. Statistical significance was determined using repeated measures one-way ANOVA; the symbol 'a' denotes statistical significance compared with IL1B ($P \leq 0.05$), while the symbol 'b' denotes statistical significance compared with TNF ($P \leq 0.05$)

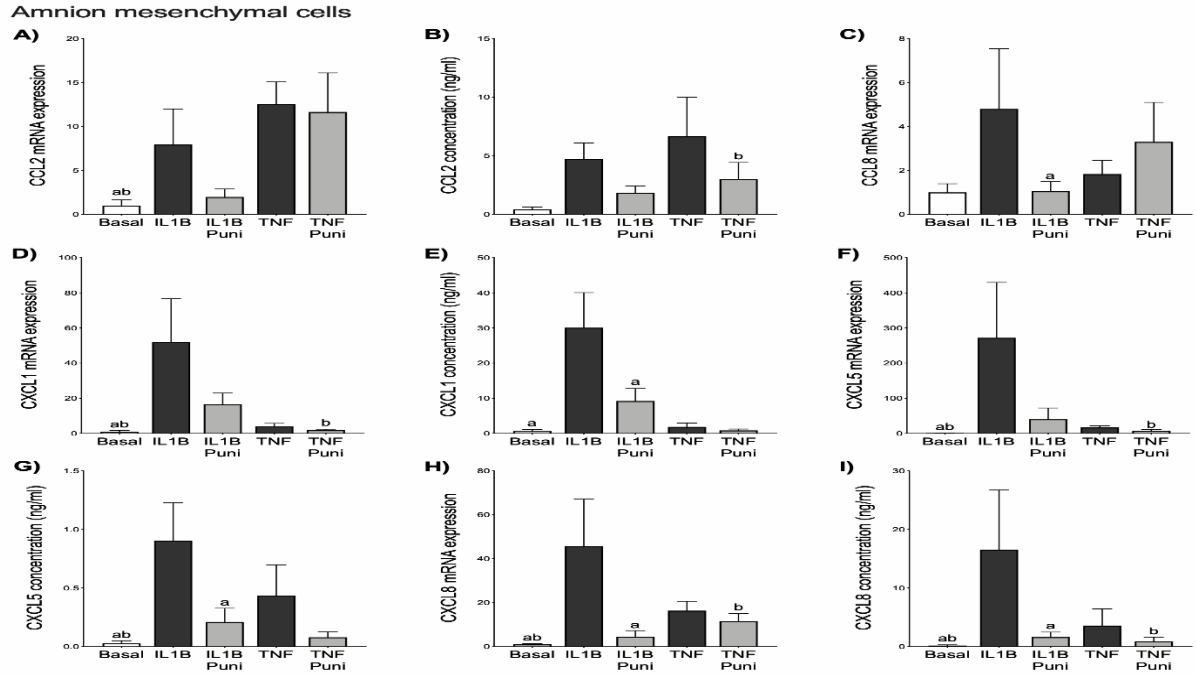

Fig. 4: Effect of punicalagin on pro-inflammatory chemokine expression and secretion in primary amnion cells. Human primary amnion cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF with or without 10 μM punicalagin (Puni) for 20 h (n = 5 patients). **(A,C,D,F,H)** CCL2, CCL8, CXCL1, CXCL5 and CXCL8 mRNA expression were analysed by RT-qPCR. (B,E,G,I) CCL2, CXCL1, CXCL5 and CXCL8 concentrations in the incubation medium were assayed by ELISA. All data is displayed as mean ± SEM. Statistical significance was determined using repeated measures one-way ANOVA; the symbol 'a' denotes statistical significance compared with IL1B ($P \leq 0.05$), while the symbol 'b' denotes statistical significance compared with TNF ($P \leq 0.05$)

Effect of Punicalagin on the Prostaglandin Pathway in Primary Myometrial and Decidual Cells

Myometrial contractions are mediated through the prostaglandin pathway, where PTGS2 is the rate-limiting factor for the production of prostaglandins, such as $PGF_{2\alpha}$ (Olson, 2003). Prostaglandins induce myometrial contractility by binding with its Prostaglandin F Receptor (PTGFR) (Olson, 2003) and contribute to the propagation of the pro-inflammatory cycle (Leimert *et al*., 2019). In this study, IL1B and TNF significantly increased PTGS2 mRNA expression and $PGF_{2\alpha}$ concentration in primary human myometrial cells compared with basal (Fig. 5A, B). Punicalagin significantly blunted IL1B-induced PTGS2 mRNA expression and IL1B- and TNF-induced $PGF_{2\alpha}$ secretion (Fig. 5A, B). There was, however, no effect of punicalagin on TNF-induced PTGS2 mRNA expression (Fig. 5A).

Decidua is also known to contribute to production of prostaglandins which can induce myometrial contractions (Skinner and Challis, 1985). In this study, IL1B significantly upregulated $PGF_{2\alpha}$ secretion in primary human decidual cells compared with basal, but there was no effect of TNF on $PGF_{2\alpha}$ secretion (Fig. 5D). There was also no effect of IL1B or TNF on PTGS2 mRNA expression (Fig. 5C). Punicalagin treatment significantly blocked both IL1B- and TNF-induced $PGF_{2\alpha}$ concentration (Fig. 5D); however, there was no effect of punicalagin on PTGS2 mRNA expression (Fig. 5C).

Effect of Punicalagin on Myometrial Cell Contractility

Given the effects of punicalagin on the prostaglandin pathway, we explored the effect of punicalagin on myometrial cell contractility using a 3D collagen gel assay (Fig. 6). Primary human myometrial cells were embedded in collagen gels and stimulated with TNF with or without punicalagin treatment. TNF significantly reduced collagen gel area compared with basal by 35.8%, indicating increased myometrial cell contraction. Punicalagin co-treatment reversed collagen gel shrinkage by 103.9% compared with TNF, suggesting that punicalagin blocked myometrial cell contraction.

Effect of Punicalagin on Matrix Metalloproteinases in Primary Decidual and Amnion Cells

Matrix metalloproteinases are enzymes involved in remodelling and degradation of the extracellular matrix in the fetal membranes. Inflammation stimulates production of enzymes, such as MMP1, MMP3, MMP9 and MMP13, that target various cell populations in the fetal membranes to induce weakening and rupture of the fetal membranes (Weiss *et al*., 2007). Thus, we sought to investigate the effect of punicalagin on the mRNA expression of MMP1, MMP3, MMP9 and MMP13 in primary decidual and amnion mesenchymal cells (Fig. 7).

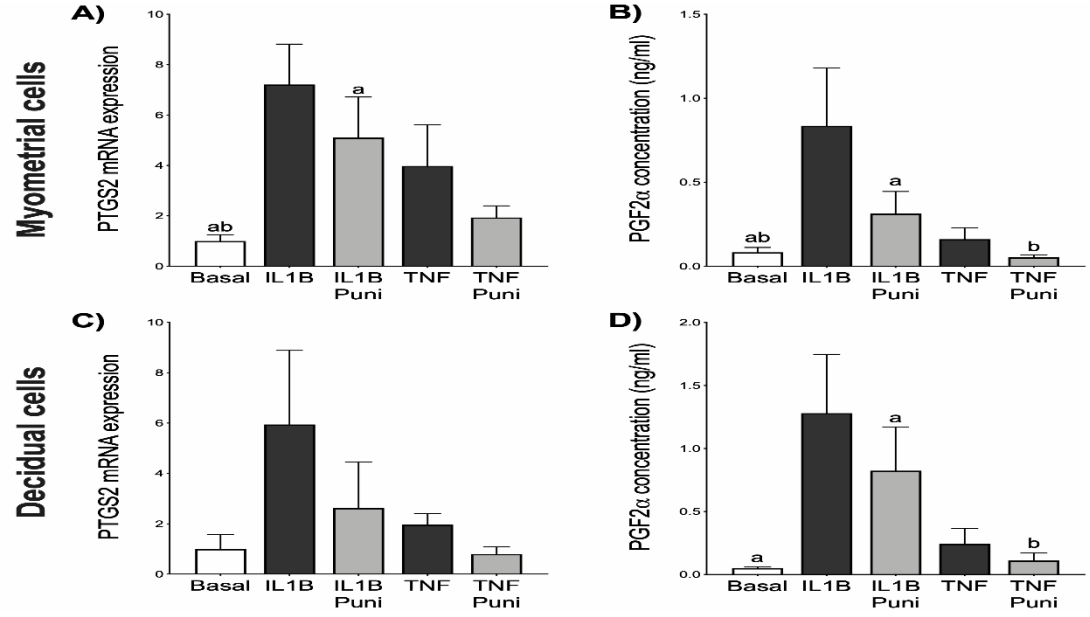

Fig. 5: Effect of punicalagin on COX-2-prostaglandin pathway proteins in primary myometrial and decidual cells. Human primary (A,B) myometrial and (C,D) decidual cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF with or without 10 μm punicalagin (Puni) for 20 h (n = 5 patients). (A, C) PTGS2 mRNA expression was analysed by RT-qPCR. (B, D) $PGF_{2\alpha}$ concentrations in the incubation media were analysed by ELISA. All data is displayed as mean± SEM. Statistical significance was determined using repeated measures one-way ANOVA; the symbol 'a' denotes statistical significance compared with IL1B ($P \leq 0.05$), while the symbol 'b' denotes statistical significance compared with TNF ($P \leq 0.05$)

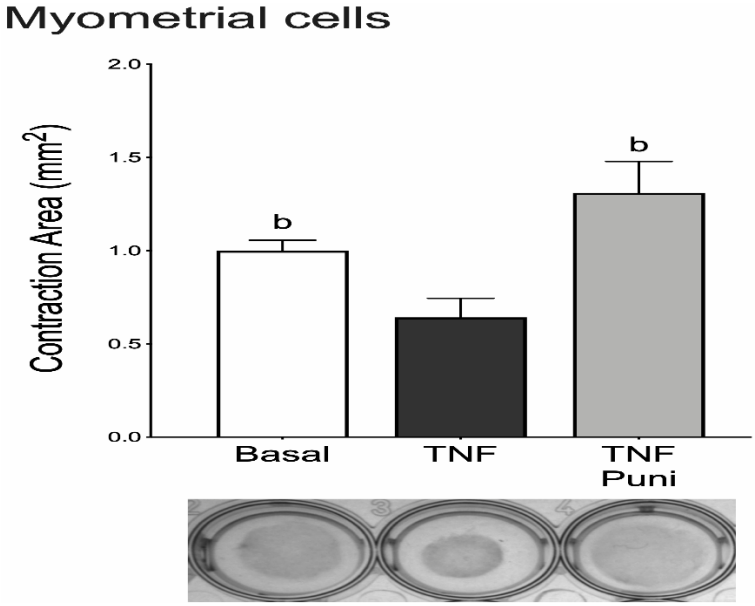

Fig. 6: Effect of punicalagin on human primary myometrial cell contraction. Collagen gel cell contraction assays were performed using 3D collagen gels embedded with human primary myometrial cells and incubated in 10 ng/mL TNF, with or without 10 µm Puni for 50 h (n = 4 patients). The area of the gel was determined using Chemidoc MP. Representative gel contraction image from 1 patient is also shown. All data is displayed as mean ± SEM. Statistical significance was determined using repeated measures one-way ANOVA; the symbol 'b' denotes statistical significance compared with TNF ($P \leq 0.05$)

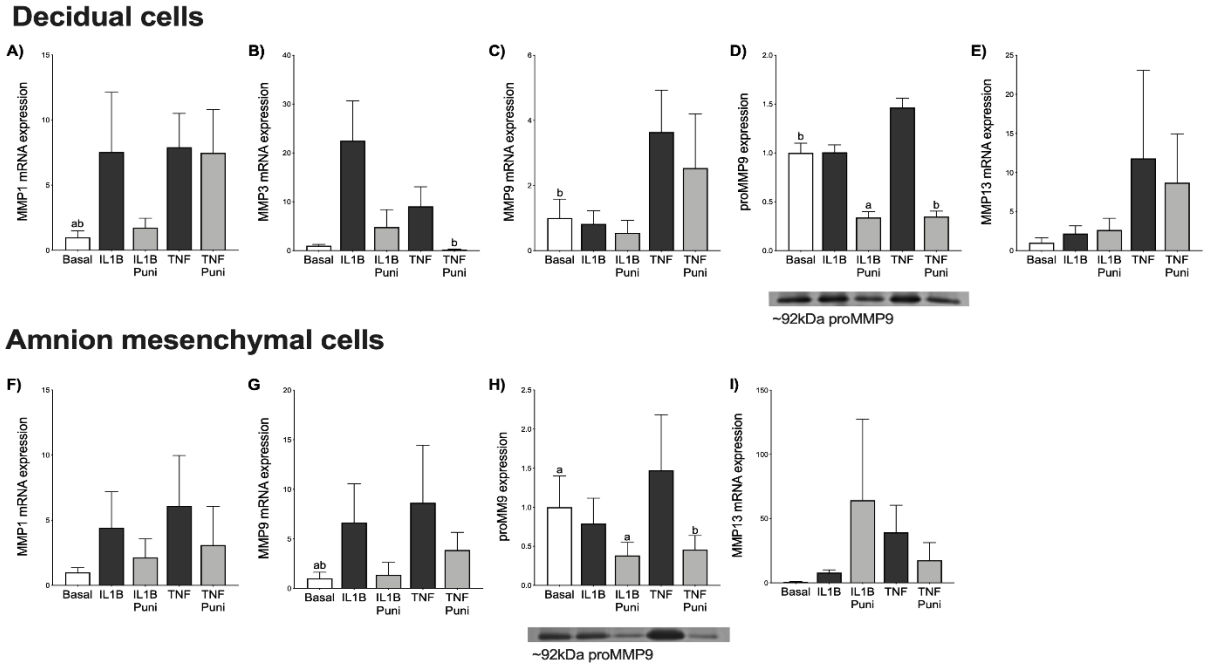

Fig. 7: Effect of punicalagin on MMP expression and activity in primary decidual and amnion cells. Human primary **(A-E)** decidual and **(F-I)** amnion cells were incubated in 1 ng/mL IL1B or 10 ng/mL TNF with or without 10 µM punicalagin (Puni) for 20 h (n = 5 patients). (A,F) MMP1, (B) MMP3, (C,G) MMP9 and (E,I) MMP13 mRNA expression was analysed by RT-qPCR. (D,H) ProMMP9 expression in the incubation media was assessed by gelatin zymography. Representative gel image from 1 patient/tissue is also shown. All data is displayed as mean ± SEM. Statistical significance was determined using repeated measures one-way ANOVA; the symbol 'a' denotes statistical significance compared with IL1B ($P \leq 0.05$), while the symbol 'b' denotes statistical significance compared with TNF ($P \leq 0.05$)

In primary decidual cells, IL1B significantly increased MMP1 mRNA expression compared with basal (Fig. 7A). TNF significantly also increased MMP1 and MMP9 mRNA expression and proMMP9 protein secretion compared with basal (Fig. 7A,C,D). There was no effect of IL1B on MMP3, MMP9, MMP13 mRNA expression and proMMP9 expression compared with basal (Fig. 7B-E). There was also no effect of TNF on MMP3 or MMP13 mRNA expression compared with basal (Fig. 7B,E). Punicalagin significantly decreased MMP3 mRNA expression in the presence of TNF (Fig. 7B) and IL1B- and TNF-induced proMMP9 expression (Fig. 7D). There was, however, no effect of punicalagin on IL1B-induced MMP1, MMP3, MMP9 or MMP13 mRNA expression (Fig. 7A-C,E), or TNF-induced MMP1, MMP9 or MMP13 mRNA expression (Fig. 7A,C,E).

In primary amnion mesenchymal cells, IL1B significantly upregulated MMP9 mRNA expression and downregulated proMMP9 expression compared with basal (Fig. 7G,H), while TNF significantly increased MMP9 mRNA expression (Fig. 7G). There was no effect of IL1B or TNF on MMP1 or MMP13 mRNA expression (Fig. 7F,I). Punicalagin treatment significantly supressed proMMP9 protein secretion in the presence of IL1B or TNF (Fig. 7H). There was no effect of punicalagin on MMP1, MMP9 or MMP13 mRNA expression (Fig. 7F,G,I). MMP3 mRNA expression in amnion mesenchymal cells was non-detectable.

Discussion

To our knowledge, this is the first study to report that punicalagin suppresses expression of pro-inflammatory and pro-labor molecules in cells isolated from human myometrium and fetal membranes (refer Supplementary Table 1 for summary of results). In primary myometrial, decidual and amnion cells, punicalagin was able to block inflammation-induced increases in pro-inflammatory cytokines and chemokines. In primary myometrial and decidual cells, punicalagin suppressed the expression of $PGF_{2\alpha}$, which is involved in activating myometrial contractility. In support, functional studies demonstrated that punicalagin blocked inflammation-induced myometrial cell contractility. In cells isolated from the fetal membranes, punicalagin downregulated the expression of the ECM-degrading enzyme proMMP9. Collectively, these data suggest that punicalagin suppresses expression of pro-inflammatory and pro-labor mediators involved in labor onset and progression in human gestational tissues *in vitro*.

Inflammation plays a role in the pathophysiology of spontaneous preterm birth (Kim *et al*., 2013, Wenstrom *et al*., 1998). Pro-inflammatory cytokines such as IL1B and TNF induce production of pro-inflammatory mediators and propagate a pro-inflammatory cycle in these gestational tissues (Lappas, 2017, Lim *et al*., 2017, Lappas *et al*., 2006). This inflammatory environment can then lead to downstream production of other pro-labor mediators to induce myometrial contractility (Rauk and Chiao, 2000) and fetal membrane rupture (Kumar *et al*., 2006). In this study, punicalagin significantly blocked inflammation-induced increases in pro-inflammatory cytokine mRNA expression and secretion, as well as chemokine protein secretion. Pro-inflammatory cytokines, such as IL1B, have previously been demonstrated to stimulate expression of contraction-associated proteins, uterotonics and ECM-degrading and -remodelling enzymes in human myometrial cells (Lappas, 2017). Similarly, IL1A, IL6 and GM-CSF are central to propagation of inflammation in preterm birth, as well as inducing uterine activation and fetal membrane weakening (Farina and Winkelman, 2005; Christiaens *et al*., 2008, Kumar *et al*., 2014). By downregulating mRNA expression and secretion of pro-inflammatory cytokines, punicalagin may then be able to prevent additional production of pro-inflammatory cytokines and chemokines, as well as downstream effects on myometrial contractility. Punicalagin was also noted to exert cell-specific effects on pro-inflammatory cytokine and chemokine expression and secretion; varying dosages and incubation times may elicit more comparable effects in each cell type. Interestingly, while punicalagin reduced protein secretion of most chemokines in myometrial, decidual and amnion cells, there was a weaker effect against their mRNA expression (for example, CCL2, CXCL1 and CXCL5). This may suggest that punicalagin regulates inflammatory secretion at a post-transcriptional level, rather than at the mRNA level. Regardless, by suppressing chemokine secretion into the incubation media, punicalagin may also limit leukocyte infiltration into intrauterine tissues, which is a known feature of laboring myometrium and fetal membranes (Hamilton *et al*., 2012, Thomson *et al*., 1999). Together, these findings suggest that punicalagin may be able to stem the propagation of pro-inflammatory mediators in myometrium and fetal membranes. The anti-inflammatory potential of punicalagin may then be able to block downstream effects on myometrial contractility and rupture of fetal membranes.

Myometrial contractility is mediated by way of contraction-associated proteins and the uterotonic $PGF_{2\alpha}$ (Roy *et al*., 2020). PTGS2 is the enzyme responsible for the production of $PGF_{2\alpha}$ in both myometrium (Olson, 2003) and decidua (Skinner and Challis, 1985). $PGF_{2\alpha}$ can then induce myometrial contractility by binding with PTGFR (Olson, 2003) to induce cytoskeletal changes for contractility (Roy *et al*., 2020), as well as contribute to the production of pro-inflammatory mediators (Leimert *et al*., 2019). Previous studies found that $PGF_{2\alpha}$ treatment of pregnant mice induced early labor and abortion (Sugimoto *et al*., 1997, Shaw, 1983), while a PTGFR knockout model delayed parturition (Harper and Skarnes, 1972).

Table 1: Summary of effects of punicalagin on IL1B- and TNF-treated primary human myometrial, decidual and amnion mesenchymal cells *in vitro*

	Primary myometrial cells		Primary decidual cells		Primary amnion mesenchymal cells	
	Response to IL1B	Response to TNF	Response to IL1B	Response to TNF	Response to IL1B	Response to TNF
Pro-inflammatory cytokines						
IL1A mRNA	↓	↓	-	↓	-	↓
IL1B mRNA	NA	-	NA	↓	NA	↓
IL6 mRNA	↓	-	-	-	-	-
IL6 concentration	↓	↓	↓	↓	-	-
GM-CSF mRNA	↓	-	-	-	-	-
Pro-inflammatory chemokines						
CCL2 mRNA	-	-	-	-	-	-
CCL2 concentration	↓	↓	↓	↓	-	↓
CCL8 mRNA	-	-	-	-	↓	-
CXCL1 mRNA	-	-	-	-	-	↓
CXCL1 concentration	↓	↓	↓	↓	↓	-
CXCL5 mRNA	-	-	-	-	-	↓
CXCL5 concentration	↓	↓	↓	↓	↓	-
CXCL8 mRNA	↓	↓	↓	↓	↓	↓
CXCL8 concentration	↓	↓	↓	↓	↓	↓
Prostaglandin pathway proteins						
PTGS2 mRNA	↓	-	-	-	NA	NA
PGF2 concentration	↓	↓	↓	↓	NA	NA
Matrix metalloproteinases						
MMP1 mRNA	NA	NA	-	-	-	-
MMP3 mRNA	NA	NA	-	↓	NA	NA
MMP9 mRNA	NA	NA	-	-	-	-
proMMP9 expression	NA	NA	↓	↓	↓	↓
MMP13 mRNA	NA	NA	-	-	NA	NA

Legend
↓ significantly decreased compared to IL1B- or TNF-treated cells
↑ significantly increased compared to IL1B- or TNF-treated cells
- no significant difference compared to IL1B- or TNF-treated cells
NA Not assessed

In this study, punicalagin suppressed inflammation-induced PTGS2 mRNA expression and $PGF_{2\alpha}$ secretion in myometrial cells. Punicalagin also reduced inflammation-induced $PGF_{2\alpha}$ secretion, but not PTGS2 mRNA expression, from decidual cells. This may suggest that punicalagin acts in a tissue-dependent manner to inhibit PGF2α secretion – while punicalagin may target PTGS2 expression in myometrial cells, punicalagin may act on downstream enzymes, such as PGF synthases, in decidua cells. Importantly, punicalagin significantly prevented shrinkage of myometrial cell-embedded collagen gels in the presence of inflammation, indicating that punicalagin can suppress myometrial cell contractility. These data suggest that punicalagin targets the prostaglandin pathway to suppress prostaglandin secretion and inhibit myometrial contractility. This indicates that punicalagin may possess tocolytic properties and may be able to inhibit myometrial contractility associated with preterm birth.

Preterm premature rupture of membranes occurs through the weakening and rupture of the fetal membranes (Lannon *et al*., 2014). Matrix metalloproteinases are enzymes involved in ECM-remodelling and degradation that lead to weakening of the fetal membranes (Weiss *et al*., 2007). In amniotic fluid and maternal plasma, elevated levels of MMP1, MMP3 and MMP13 are associated with preterm birth (Park *et al*., 2003; Maymon *et al*., 2000; Soydinç *et al*., 2012; Fortunato *et al*., 1999). In fetal membranes, MMP9 protein expression is increased during and after labor (Tsatas *et al*., 1999), particularly localised to the site of rupture (Chai *et al*., 2013). Further, there is an inverse relationship between MMP9 and fetal membrane strength (El Khwad *et al*., 2005). These studies suggest a major contributing role for MMP9 in fetal membrane degradation. In this study, punicalagin downregulated MMP3 mRNA expression in decidual cells and proMMP9 expression in decidual and amnion mesenchymal cells. By targeting proMMP9 expression, punicalagin may be able to limit MMP9-associated fetal membrane weakening. Further, punicalagin inhibited proMMP9 expression, but not MMP1, MMP9 or MMP13 mRNA expression, suggesting that punicalagin may preferentially target protein expression or enzyme activity

of MMPs. Additional studies exploring the effect of punicalagin on MMP protein expression and enzyme activity in the fetal membranes would provide further insight to the effect of punicalagin on fetal membrane weakening associated with preterm birth.

Collectively, these data show that punicalagin prevents expression of pro-inflammatory and pro-labor mediators in primary human gestational tissues. These findings suggest punicalagin may play a role in preventing intrauterine inflammation associated with human preterm birth. In a supplementary pilot study, pregnant mice were pre-treated with punicalagin for three days, prior to LPS administration to induce a pro-inflammatory intrauterine environment (Supplementary Fig. 1). These preliminary experiments show punicalagin can suppress expression of pro-inflammatory cytokines and chemokines in myometrium and decidua of an LPS mouse model of intrauterine inflammation (Supplementary Fig. 1). However, further research is required to effectively assess the impact of punicalagin on circulating levels of pro-inflammatory mediators, on prostaglandin-pathway proteins and MMP expression in an *in vivo* mouse model of intrauterine inflammation. Furthermore, the effect of punicalagin on delaying LPS-induced labor in pregnant mice remains unknown and warrants future research.

Intrauterine inflammation not only contributes to the pathophysiology of preterm birth, but has also been evidenced to alter fetal neuronal morphology in a mouse model of inflammation-induced preterm birth (Burd *et al*., 2010). It is of note, that previous studies have demonstrated a role for punicalagin and pomegranate juice in improved fetal neurodevelopmental outcomes. For example, a rat model of inflammation-induced fetal brain injury showed that pomegranate juice supplementation reduced fetal brain inflammation (Ginsberg *et al*., 2018). A recent human clinical trial in pregnant women with intrauterine growth restriction also found that, compared with a polyphenol-free placebo, pomegranate juice supplementation altered infant neural structure, in a manner that was indicative of improved maturation and neuroprotection (Matthews *et al*., 2019). These findings, together with the data presented in this study, may suggest that punicalagin may not only inhibit intrauterine inflammation, but may also contribute to fetal neuroprotection. Collectively, these studies suggest polyphenols may represent a novel strategy for targeted suppression of inflammation underlying preterm birth.

A number of strengths and limitations exist in this investigation. Firstly, the findings of the study are strengthened by using three distinct primary human cell lines derived from the critical tissues involved in preterm birth, including the myometrium and the fetal membranes (decidua and amnion mesenchyme). Secondly, the effect of punicalagin in vitro was measured in response to two different pro-inflammatory stimuli (IL1B and TNF), which each induce inflammation through distinct mechanisms (Lappas, 2017, Lim *et al*., 2017). While punicalagin suppressed intrauterine inflammation in pregnant mice, further studies are needed to determine if punicalagin can delay LPS-induced preterm labor in pregnant mice, as well as to identify the specific effects of punicalagin on fetal neurodevelopmental outcomes. Additional data on the effect of punicalagin on MMP protein secretion and activity would further complement the mRNA expression findings in this study. Finally, further investigation of the effect of punicalagin on mechanistic pathways, such as Akt/MAPK/NF-κB pathways may further strengthen the anti-inflammatory findings presented in this study.

Spontaneous preterm birth is the leading cause of neonatal morbidity and mortality; despite this, current intervention therapeutics benefit some, but not all, subsets of high-risk pregnancies (Beck *et al*., 2010, Haas *et al*., 2012). Here, we investigated the anti-inflammatory and anti-labor potential of punicalagin in an *in vitro* model of intrauterine inflammation associated with spontaneous preterm birth. *In vitro*, punicalagin suppressed inflammation-induced expression of pro-inflammatory cytokines, chemokines, prostaglandin pathway-associated proteins, uterotonics and MMPs involved in myometrial activation and fetal membrane rupture. Overall, this study found that punicalagin can suppress expression of pro-inflammatory and pro-labor molecules *in vitro*. Further study using an *in vivo* animal model is warranted to determine the efficacy of punicalagin in delaying preterm labor.

Acknowledgements

The following are gratefully acknowledged: Dr. Ratana Lim for her assistance with the *in vivo* mouse study, the clinical Research Midwives Gabrielle Pell, Genevieve Christophers and Rachel Murdoch for sample collection; the Obstetrics and Midwifery staff of the Mercy Hospital for Women for their co-operation; the women who generously donated their tissue samples to the study and the Austin BioResource Facility staff for the breeding, housing and maintenance of the mice.

Funding

This study was supported by project grants from the Norman Beischer Medical Research Foundation and the Austin Medical Research Foundation.

Author Contributions

Martha Lappas: Designed the study and critically reviewed the manuscript.

Caitlyn Nguyen-Ngo: Conducted the experiments, performed data analysis and wrote the initial draft of the manuscript.

Jane C Willcox: Critically reviewed the study and the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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