Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2013, Article ID 485201, 10 pages
http://dx.doi.org/10.1155/2013/485201

Research Article

Dietary Flavonoids as Therapeutics for Preterm Birth: Luteolin and Kaempferol Suppress Inflammation in Human Gestational Tissues *In Vitro*

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Received 19 April 2013; Accepted 20 May 2013

Academic Editor: Tullia Maraldi

Infection/inflammation is commonly associated with preterm birth (PTB), initiating uterine contractions and rupture of fetal membranes. Proinflammatory cytokines induce matrix metalloproteinases (MMPs) that degrade the extracellular matrix (ECM) and prostaglandins which initiate uterine contractions. Nuclear factor-κB (NF-κB) and activator-protein- (AP-)1 have key roles in the formation of these prolabour mediators. In nongestational tissues, dietary flavonoids such as luteolin and kaempferol inhibit NF-κB, AP-1, and their downstream targets. The aim of this study was to determine if luteolin and kaempferol reduce infection-induced prolabour mediators in human gestational tissues. Fetal membranes were incubated with LPS, and primary amnion cells and myometrial cells were incubated with IL-1β in the absence or presence of luteolin or kaempferol. Luteolin and kaempferol significantly reduced LPS-induced secretion of proinflammatory cytokines (IL-6 and IL-8) and prostaglandins (PGE_2 and $PGF_{2\alpha}$) in fetal membranes, IL-1β-induced COX-2 gene expression and prostaglandin production in myometrium, and IL-1β-induced MMP-9 activity in amnion and myometrial cells. Luteolin and kaempferol decreased IL-1β-induced NF-κB p65 DNA binding activity and nuclear c-Jun expression. In conclusion, luteolin and kaempferol inhibit prolabour mediators in human gestational tissues. Given the central role of inflammation in provoking preterm labour, phytophenols may be a therapeutic approach to reduce the incidence of PTB.

1. Introduction

Preterm birth (PTB) is the single most important complication contributing to poor pregnancy and neonatal outcome, globally, being defined as childbirth occurring at less than 37-week gestation. According to the World Health Organisation (WHO), more than 1 in 10 babies is born preterm every year [1], and this incidence has been steadily rising since the 1980s despite obstetric intervention [2]. With long term health consequences, the care of the prematurely born infant is extremely expensive, and the emotional stress on the family is sizeable. The estimated cost associated with PTB in the United States alone, in terms of medical and educational expenditure and lost productivity in 2005, was more than US$26.2 billion [3].

Spontaneous PTB (sPTB) accounts for approximately 70% of all PTB with 60% due to idiopathic preterm labour and the remaining 40% due to preterm pre-labour rupture of the fetal membranes (PPROM) [4]. Infection is the biggest aetiological factor for the onset of sPTB [5]. Infection activates the maternal immune system, which causes production of the proinflammatory cytokines IL-1β and TNF-α. They bind to their respective receptors located on placenta, fetal membranes, and myometrium to induce the activity of the proinflammatory and prolabour transcription factors activator-protein- (AP-)1 and nuclear factor kappa B (NF-κB) [6–9]. Once activated, AP-1 and NF-κB upregulate transcription of proteases, prostaglandins, and cytokines which lead to delivery of the baby by causing cervical ripening, rupture of fetal membranes, and uterine contractions, three critical stages in the initiation of labour.

Epidemiological studies have revealed that a diet rich in plant-derived foods has a protective effect on human health [10, 11]. Luteolin is a citrus flavonoid found in high amounts in

parsley, thyme, peppermint, basil, herb, celery, and artichoke. Kaempferol is a flavonoid found in many edible plants including tea, broccoli, cabbage, kale, beans, endive, leek, tomato, strawberries, and grapes. Previous studies performed in nongestational tissues have demonstrated that luteolin and kaempferol are two phytophenols that have potent anti-inflammatory properties, exerting these actions via inhibition of NF-κB [12–15] and AP-1 [14, 16, 17]. The effect of luteolin and kaempferol as modulators of the inflammatory response associated with labour is, however, not yet known. Thus, the aim of this study is to determine whether luteolin and kaempferol will reduce infection-induced prolabour mediators in human gestational tissues. Experiments will be performed in the presence of LPS or IL-1β as these are likely to be factors stimulating proinflammatory cytokines, prostaglandins, and proteases in the context of infection-induced PTB. We analysed the effect of luteolin and kaempferol on (i) COX-2 and subsequent PGE_2 and $PGF_{2\alpha}$ production, (ii) proinflammatory cytokine (IL-6) and chemokine (IL-8) mRNA expression and release, and (iii) matrix-metalloproteinase- (MMP-)9 mRNA expression and release. The effect of luteolin and kaempferol on NF-κB (NF-κB p65 DNA binding activity and IκB-α expression) and AP-1 (nuclear c-Jun expression) was also examined.

2. Methods and Materials

2.1. Tissue Collection. Human placentae and attached fetal membranes were obtained (with Institutional Research and Ethics Committee approval) from nonobese women who delivered single, healthy infants at term gestational age (37–40-week gestation) undergoing elective Caesarean section, whether due to a medical or obstetrical reason or on patients request. Human myometrium was obtained from the upper margin of the incision made in the lower uterine segment at the time of term Caesarean section. Amnion and underlying choriodecidua were obtained 2 cm from the periplacental edge. None of the patients were in labour or had received uterotonics or tocolytics.

2.2. Fetal Membrane Explants. Fetal membranes (combined amnion and choriodecidua) were obtained within ten minutes of delivery, and dissected fragments were placed in ice-cold PBS. Tissue fragments were placed in Roswell Park Memorial Institute (RPMI) 1640 media at 37°C in a humidified atmosphere of 8% O_2 and 5% CO_2 for 1 h. Explants were blotted dry on sterile filter paper and transferred to 24-well tissue culture plates (200 mg wet weight/well). The explants were incubated, in duplicate, in 2 mL RPMI 1640 containing penicillin G (100 U/mL) and streptomycin (100 μg/mL). Explants were incubated, for 24 h, in the presence of 10 μg/mL LPS (to facilitate the production of proinflammatory mediators) in the absence (DMSO control) or presence of 20 μM luteolin or 100 μM kaempferol (Sigma, St. Louis, MO, USA) (n = 8 patients). A dose response was used to determine the concentrations of luteolin and kaempferol used for this study (data not shown), with the initial concentrations for the dose response determined from past studies [18–23]. After 24 h incubation, medium was collected and stored at −80°C until assayed for cytokine and prostaglandin concentrations as detailed below. Tissue was collected and stored at −80°C until assayed for gene expression by qRT-PCR. Experiments were performed in fetal membranes from eight patients.

2.3. Myometrial Cell Culture. Primary myometrial smooth muscle cells were used to investigate the effects of luteolin and kaempferol on the COX-prostaglandin pathway and MMP-9. Myometrial tissue was washed in PBS and finely dissected. Myometrium was minced and digested for 45 min in Dulbecco's Modified Eagle's Medium: nutrient mixture F12 (DMEM/F12) with 3 mg/mL type 1 collagenase (Worthington Biochemical, Freehold, NJ, USA) and 80 μg/mL DNase I (Roche Diagnostics Australia). Cells were centrifuged at 400× g for 10 min and grown in a 75 cm^2 flask in DMEM/F12 with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (37°C and 5% CO_2 and 21% O_2). Myometrial cells from passages 1–4 were trypsinised in TrypLE Express (Life technologies, Grand Island, NY, USA) and cultured in 12-well plates in DMEM/F12 with 10% heat inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin until they reached the required confluence (~90%). Cells were then incubated in 500 pg/mL IL-1β (to facilitate the production of proinflammatory mediators) and either in the absence (DMSO control) or presence of 20 μM luteolin or 100 μM kaempferol (n = 6 patients) for 24 h. For IκB-α studies, cells were pretreated with luteolin and kaempferol overnight, followed by a 30 min incubation with 500 pg/mL IL-1β. For c-Jun studies, cells were pretreated for 6 h with luteolin and kaempferol followed by an overnight incubation with 500 pg/mL IL-1β. The media were collected and stored at −80°C, until assayed for cytokine, prostaglandin, and MMP-9 concentrations as detailed below. Cell pellets were collected and stored at −80°C, before being analysed for IκB-α and c-Jun expression by Western blotting, gene expression by qRT-PCR, or NF-κB p65 DNA binding activity by transcription factor assay as detailed below. Experiments were performed in myometrium from six patients.

2.4. Primary Amnion Cell Culture. Primary amnion epithelial cultures were used to investigate the effects of luteolin and kaempferol on MMP-9 expression and enzyme activity. Cells were prepared as previously described [24]. Primary amnion cells (passage 1) at ~90% confluence were incubated in the absence or presence of 1 ng/mL IL-1β in the absence or presence of 20 μM luteolin or 100 μM kaempferol (n = 6 patients). After 24 h incubation, medium was collected, and assessment of enzymes of ECM weakening and rupture (MMP-9) was performed as detailed below. Cells were also collected and MMP-9 gene expression analysed by qRT-PCR as detailed below. Experiments were performed in amnion from six patients.

2.5. Cytokine and Prostaglandin Assays. Conditioned medium from cell and tissue culture experiments was assessed for IL-6 and IL-8 concentrations using commercial ELISA

according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The release of PGE_2 and $PGF_{2\alpha}$ into the incubation medium was assayed using commercially available competitive enzyme immunoassay kits according to the manufacturer's specifications (Kookaburra Kits from Sapphire Bioscience, Redfern, NSW, Australia). All data were corrected for total protein and expressed as either ng or pg per mg protein. The protein content of tissue homogenates was determined using BCA protein assay (Pierce, Rockford, USA), using BSA as a reference standard, as previously described [25–27].

2.6. Gelatin Zymography. Assessment of enzymes of ECM weakening and rupture (MMP-9) was performed by gelatin zymography as previously described [27, 28]. Proteolytic activity was remodeled as clear zones of lysis on a blue background of undigested gelatin.

2.7. RNA Extraction and qRT-PCR. Total RNA from cells and tissues was extracted using TRI Reagent according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). RNA concentrations were quantified using a spectrophotometer (NanoDrop, Thermo Scientific). RNA quality and integrity were determined via the A_{260}/A_{280} ratio. Two hundred ng (fetal membranes) or 300 ng (amnion and myometrial cells) of RNA was converted to cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was diluted fiftyfold, and 4 μL of this was used to perform RT-PCR using Sensimix Plus SYBR green (Quantace, Alexandria, NSW) and 100 nM of primers: GAPDH (QT01192646), IL-6 (QT00083720), IL-8 (QT00000322), TNF-α (QT01079561), COX-2 (QT00040586), and MMP-9 (QT00040040) (Qiagen, Germantown, MD, USA). The specificity of the product was assessed from melting curve analysis. RNA without reverse transcriptase during cDNA synthesis as well as PCR reactions using water instead of template showed no amplification. Average gene C_T values were remodeled to the average GAPDH mRNA C_T values of the same cDNA sample. Fold differences were determined using the comparative C_T method and expressed relative to basal [29].

2.8. Western Blotting. Western blotting was performed as we have previously described [25, 30]. For IκB-α protein expression, cell lysates were prepared as detailed in [25, 27]. To assess c-Jun expression, nuclear protein was extracted as we have previously described [31]. Rabbit polyclonal anti-IκB-α and rabbit polyclonal anti-c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at 1 μg/mL. Forty micrograms (IκB-α) or 5 μg (c-Jun) of protein was separated on polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to PVDF. Protein expression was identified by comparison with the mobility of protein standard. Membranes were viewed and analysed using the ChemiDoc system (Bio-Rad Laboratories, Hercules, CA, USA). For the IκB-α blot, the membranes were stripped and reprobed with β-actin (A5316; Sigma, St. Louis, MO, USA), used at 1.5 μg/mL to ensure even protein loading. For the c-Jun blot, the membrane was stained with Ponceau S to ensure even loading [32].

2.9. NF-κB p65 Transcription Factor Assay. Myometrial cells were pretreated with 20 μM luteolin and 100 μM kaempferol for 6 h, followed by 24 h treatment with 500 pg/mL IL-1β (n = 4 patients). Nuclear protein was extracted [31] and NF-κB p65 DNA binding in the nuclear protein assessed using a commercially available NF-κB p65 transcription factor ELISA according to manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA). A Bio-Rad xMark Microplate Spectrophotometer was used to read the sample absorbance at 450 nm, with data expressed as absorbance at 450 nm.

2.10. Statistical Analysis. All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Data were first logarithmically transformed, before analysed by one-way ANOVA using Tukey multiple range tests. Statistical difference was indicated by a P value of less than 0.05. Data are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Effect of Luteolin and Kaempferol on Proinflammatory Cytokines. In fetal membranes, qRT-PCR was performed to determine if luteolin and kaempferol reduced steady state levels of proinflammatory cytokines. As shown in Figure 1(a), LPS induced a significant increase in proinflammatory cytokine expression. Coincubation with luteolin or kaempferol significantly abrogated LPS-induced gene expression of TNF-α, IL-6, and IL-8. LPS induced a significantly higher concentration of both IL-6 and IL-8, when compared with basal concentration (Figure 1(a)). ELISA was then used to determine cytokine release. Coincubation with luteolin or kaempferol caused a significant reduction in LPS-induced IL-6 and IL-8 concentrations (Figure 1(b)). TNF-α concentration in the incubation media was below the limit of detection of the ELISA (data not shown).

Primary myometrial cells incubated with IL-1β were associated with significantly increased mRNA expression (Figure 1(c)) and release of IL-6 and IL-8 (Figure 1(d)). However, coincubation with luteolin or kaempferol had no effect on IL-1β-induced cytokine release or gene expression.

3.2. Effect of Luteolin and Kaempferol on the COX-Prostaglandin Pathway. Fetal membranes and myometrial cells were used to determine the effect of luteolin and kaempferol on COX-2 expression and prostaglandin levels. Experiments were performed as detailed above. When compared to basal, LPS-induced COX-2 mRNA levels (Figure 2(a)) and subsequent PGE_2 and $PGF_{2\alpha}$ (Figure 2(b)) concentrations were significantly greater in fetal membranes. Coincubation with luteolin and kaempferol significantly decreased LPS-induced PGE_2 concentration (Figure 2(b)). LPS-stimulated concentrations of COX-2 mRNA expression (Figure 2(a)) and

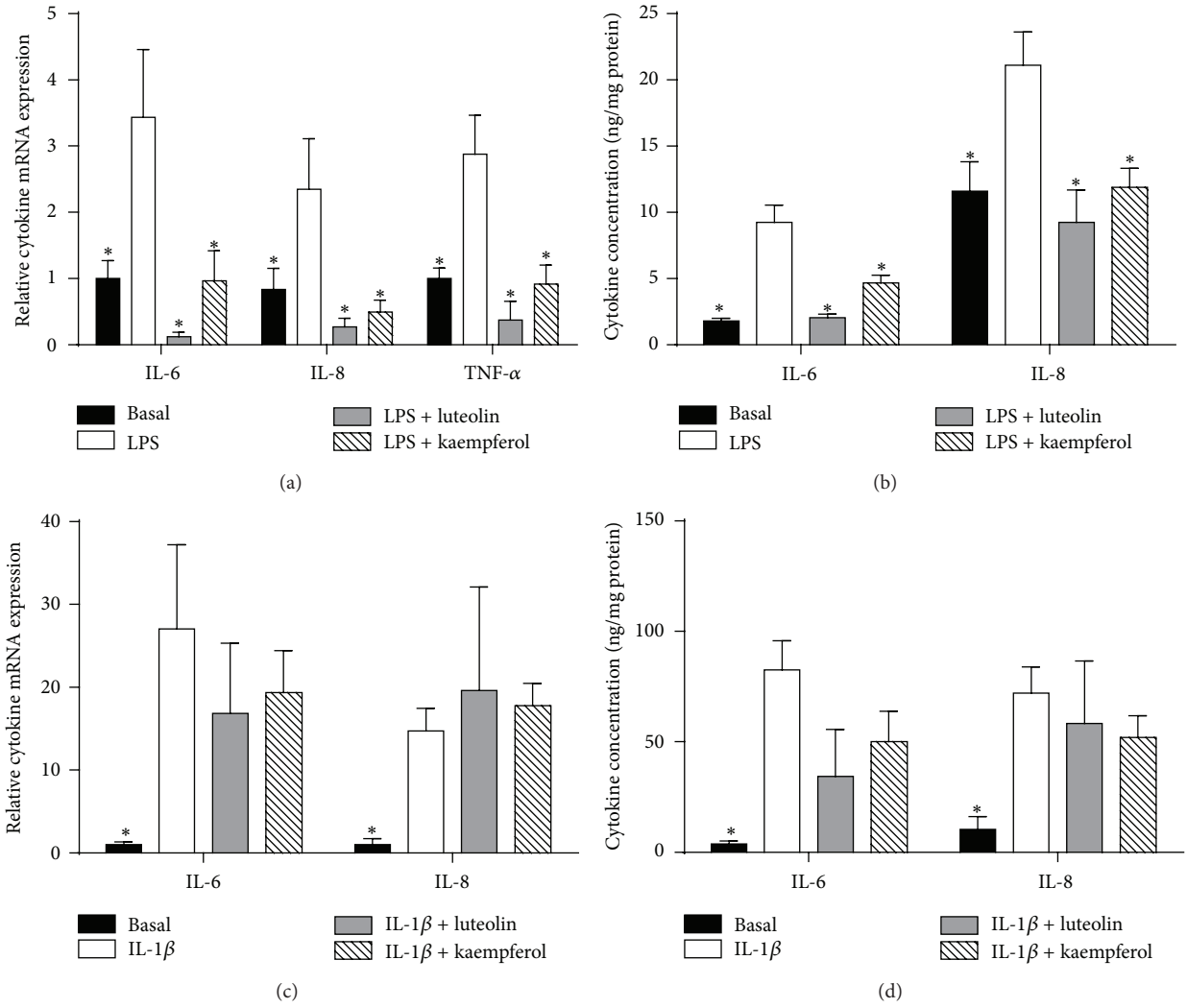

Figure 1: Effect of luteolin and kaempferol on proinflammatory cytokines. ((a), (b)) Fetal membranes were incubated with 10 μg/mL LPS in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 8$ patients). (a) TNF-α, IL-6, and IL-8 mRNA expression were quantified by qRT-PCR. (b) IL-6 and IL-8 concentrations in the conditioned media were assayed using ELISA. Each bar represents the mean ± SEM. $^*P < 0.05$ versus basal (one-way ANOVA). ((c), (d)) Primary myometrial cells were incubated with 500 pg/mL IL-1β in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 6$ patients). (c) IL-6 and IL-8 mRNA expressions were quantified by qRT-PCR. (d) IL-6 and IL-8 concentrations in the conditioned media were assayed using ELISA. Each bar represents the mean ± SEM. $^*P < 0.05$ versus basal (one-way ANOVA).

$PGF_{2\alpha}$ (Figure 2(b)) were statistically decreased by luteolin but not by kaempferol.

In myometrial cells incubated with IL-1β, COX-2 mRNA evels (Figure 2(c)) and subsequent PGE_2 and $PGF_{2\alpha}$ levels (Figure 2(d)) were significantly augmented. The addition of luteolin or kaempferol significantly attenuated IL-1β-induced PGE_2 and $PGF_{2\alpha}$ concentrations (Figure 2(d)). However, only kaempferol reduced COX-2 mRNA expression (Figure 2(c)).

3.3. Effect of Luteolin and Kaempferol on MMP-9 Expression and Activity. For amnion cells, IL-1β increased MMP-9 gene expression (Figure 3(a)) and pro-MMP-9 activity (Figure 3(b)). Coincubation with luteolin or kaempferol significantly attenuated both IL-1β-induced MMP-9 activity and expression. In myometrial cells, there was no change seen in the pro-MMP-9 bands; however there was increased active MMP-9 seen with the addition of IL-1β (Figure 3(d)). Both luteolin and kaempferol attenuated this IL-1β-induced increase in active MMP-9 activity. There was no change in MMP-9 mRNA expression with the addition of IL-1β in myometrial cells.

3.4. Effect of Luteolin and Kaempferol on NF-κB and AP-1 Transcriptional Pathways. In unstimulated cells, the NF-κB complex is made up of the p50/p65 subunits attached to IκB-α in the cytosol. While IκB-α is attached to the complex,

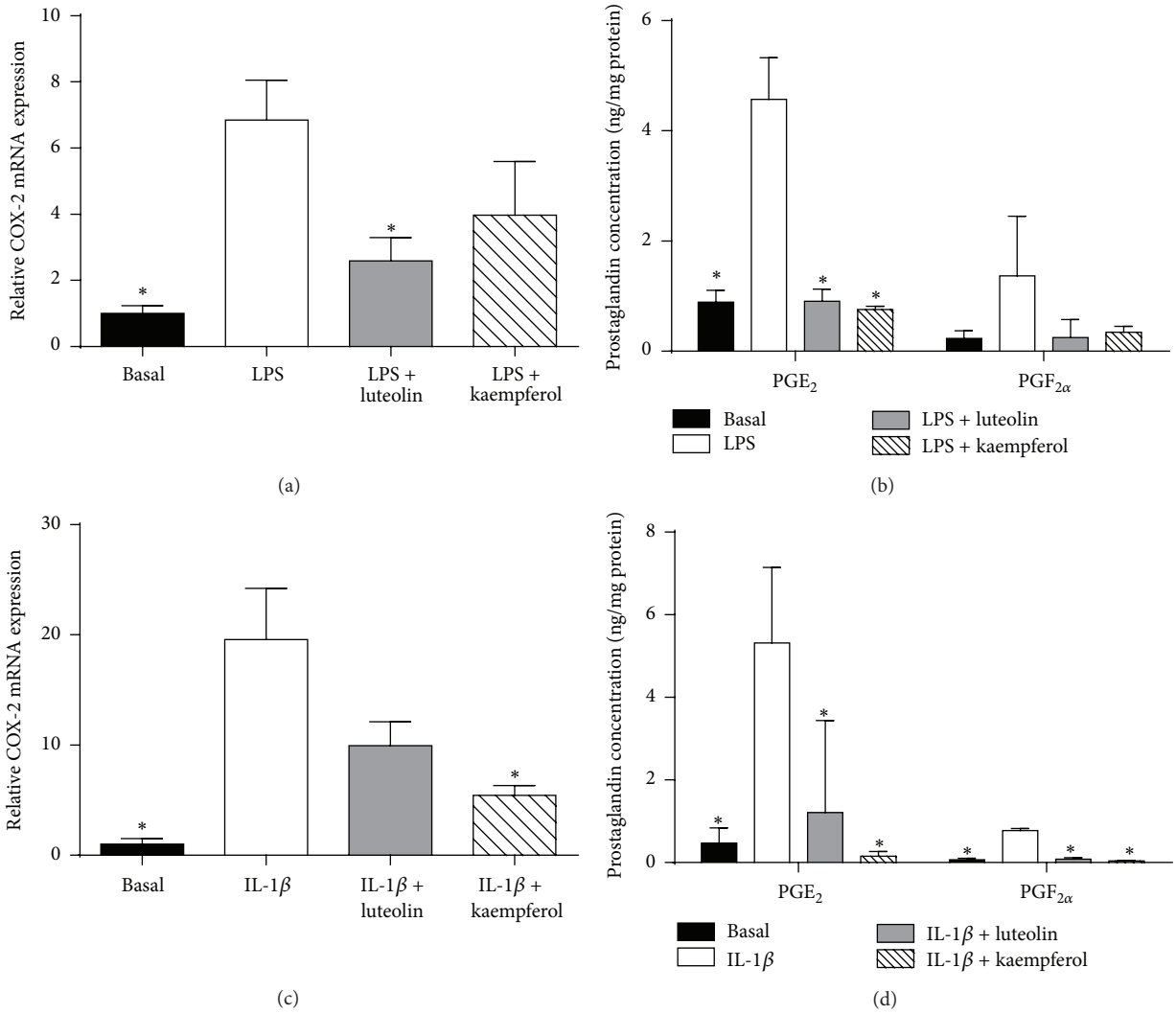

Figure 2: Effect of luteolin and kaempferol on the COX-prostaglandin pathway. ((a), (b)) Fetal membranes were incubated with 10 μg/mL LPS in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 6$ patients). (a) COX-2 mRNA expression was quantified by qRT-PCR. (b) PGE_2 and $PGF_{2\alpha}$ concentrations in the conditioned media were assayed using EIA. Each bar represents the mean ± SEM. $^*P < 0.05$ versus basal (one-way ANOVA). ((c), (d)) Primary myometrial cells were incubated with 0.5 ng/mL IL-1β in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 5$ patients). (c) COX-2 mRNA expression was quantified by qRT-PCR. (d) PGE_2 and $PGF_{2\alpha}$ concentrations in the conditioned media were assayed using EIA. Each bar represents the mean ± SEM. $^*P < 0.05$ versus basal (one-way ANOVA).

NF-κB is inactive. Activation by cytokines causes IκB-α to dissociate from the NF-κB complex. IκB-α is subsequently ubiquitinated and then degraded, allowing the NF-κB p50/p65 subunits to translocate to the nucleus [33]. The effect of luteolin and kaempferol treatment on IL-1β-induced IκB-α expression was determined in myometrial cells by Western blot analysis. Myometrial cells were pretreated with 20 μM luteolin and 100 μM kaempferol overnight, followed by 30 min 500 pg/mL IL-1β treatment ($n = 3$ patients). As expected, IL-1β induced a decrease in IκB-α expression (Figure 4(a)). However pre-treatment with either luteolin or kaempferol had no significant effect on attenuating this IL-1β-induced decrease in IκB-α expression (Figure 4(a)).

Once activated by cytokines, NF-κB enters the nucleus of cells where it binds to DNA to initiate gene transcription. There is potential for this binding to be inhibited by either blocking binding sites on the DNA itself or on NF-κB. NF-κB p65 DNA binding activity was assessed using a NF-κB p65 transcription factor assay. Nuclear protein was then extracted. IL-1β induced a significant increase in NF-κB DNA binding activity (Figure 4(b)). Co-treatment with both luteolin and kaempferol significantly attenuated IL-1β-induced NF-κB p65 DNA binding activity (Figure 4(b)).

To examine the effect of luteolin and kaempferol on AP-1, we examined c-Jun, as it forms part of the AP-1 early response transcription factor [34]. When compared to untreated cells (basal), IL-1β induced an increase in c-Jun expression (Figure 4(c)). Coincubation with luteolin and kaempferol attenuated the IL-1β-induced increase in c-Jun nuclear protein expression (Figure 4(c)).

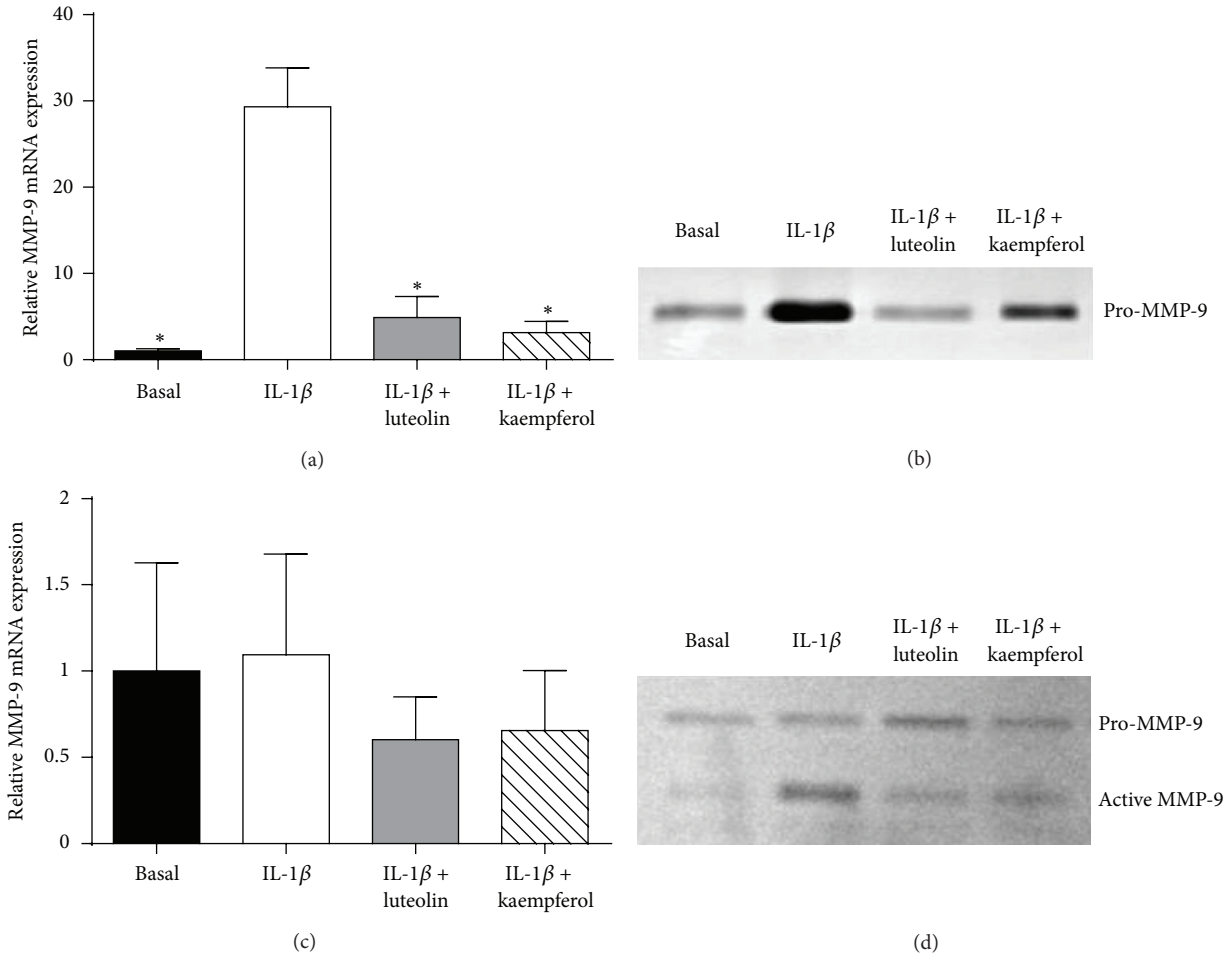

Figure 3: Effect of luteolin and kaempferol on MMP-9 expression and activity. ((a), (b)) Primary amnion cells were incubated with 1 ng/mL IL-1β in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 6$ patients). ((c), (d)) Primary myometrial cells were incubated with 500 pg/mL IL-1β in the absence or presence of 20 μM luteolin or 100 μM kaempferol for 24 h ($n = 4$ patients). ((a), (c)) MMP-9 mRNA expression was quantified by qRT-PCR. ((b), (d)) The incubation medium was assayed for MMP-9 activity by gelatin zymography. Zymography from one patient is shown.

4. Discussion

The data presented in this study demonstrate that the two dietary phytophenols luteolin (from celery) and kaempferol (from grapefruit and tea) exert anti-inflammatory properties in gestational tissues. In human fetal membranes, luteolin and kaempferol treatment attenuated LPS, or IL-1β induced increases in mRNA expression and secretion of proinflammatory cytokines, COX-2 mRNA expression and subsequent prostaglandin release, and MMP-9 expression and secretion. In myometrium cells, luteolin and kaempferol significantly decreased COX-2 expression, prostaglandin release, and MMP-9 activity induced by IL-1β. There is, however, no effect of luteolin and kaempferol on proinflammatory cytokine expression or secretion in myometrial cells. Luteolin and kaempferol were found to act via the NF-κB and AP-1 pathways, inhibiting NF-κB p65 DNA binding activity and nuclear c-Jun expression in myometrial cells.

Proinflammatory cytokines play a key role as mediators of inflammation in preterm and term labour. In human gestational tissues and amniotic fluid, these cytokines are increased with the onset of human labour at term [35], and preterm [36] and more so in the presence of infection [37]. TNF-α and IL-1β exert proinflammatory actions such as the increase of prostaglandins and ECM degrading enzymes [38]. This leads to initiation of the three critical stages of human labour: rupture of fetal membranes, cervical ripening, and uterine contractions [35, 39, 40]. In this study, we used human fetal membranes and myometrial cells to determine the effect of luteolin and kaempferol on LPS or IL-1β-induced expression and release of the proinflammatory cytokines TNF-α, IL-6, and IL-8. We found that both luteolin and kaempferol significantly reduced the mRNA expression and secretion of proinflammatory cytokines in fetal membranes. Similarly, past studies performed in nongestational tissues have reported such anti-inflammatory actions for luteolin and kaempferol [12, 15–17]. However, it was found that luteolin and kaempferol had no effect on cytokine expression or secretion from pregnant myometrial cells. It is possible that longer

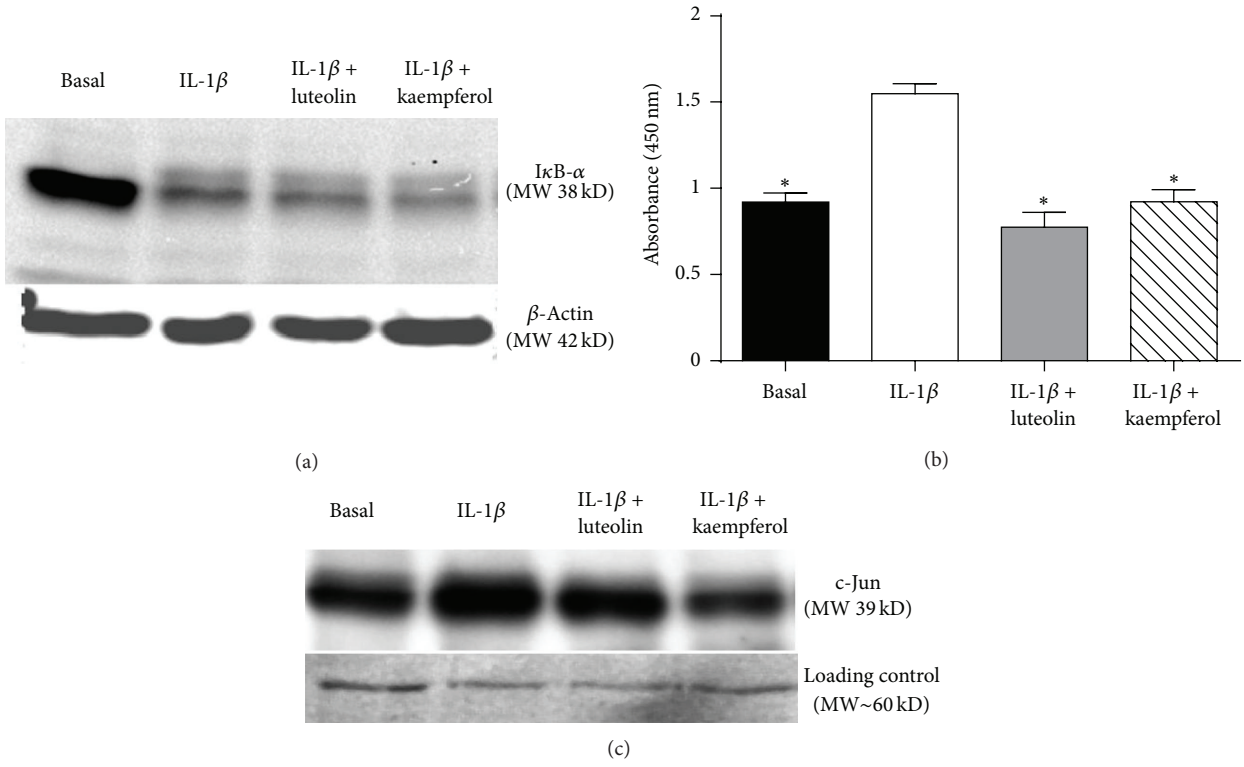

Figure 4: Effect of luteolin and kaempferol treatment on NF-κB DNA binding activity and c-Jun expression. ((a), (c)) Primary myometrial cells were pretreated with 20 μM luteolin or 100 μM kaempferol for 24 h, followed by 30 min incubation with 500 pg/mL IL-1β. (a) Representative IκB-α Western blot from one patient using β-actin as a loading control. Similar data were obtained for another two patients. (b) Primary myometrial cells were pretreated with 20 μM luteolin or 100 μM kaempferol for 6 h, followed by treatment with 500 pg/mL IL-1β for 24 h. NF-κB p65 DNA binding activity was assayed using a NF-κB p65 transcription factor assay ($n = 4$ patients). Data is displayed as absorbance at 450 nm. Each bar represents the mean ± SEM. $^*P < 0.05$ versus IL-1β-induced NF-κB DNA binding (one-way ANOVA). (c) Representative Western blot of nuclear c-Jun from one patient using Ponceau S as a loading control. Similar data were obtained for another four patients.

or shorter incubation times or higher concentrations of phytophenols are required to elicit a protective response in myometrial cells.

COX-2 stimulates production of prostaglandins, which are important in the initiation and maintenance of labour, by increasing uterine contraction and promoting cervical ripening and the decidual-fetal membrane activation [38, 41]. Luteolin and kaempferol have been demonstrated in nongestational tissues to cause inhibition of COX-2, hence causing inhibition of prostaglandins [42, 43]. In this study, we demonstrate that luteolin and kaempferol significantly decreased LPS, IL-1β-induced COX-2 gene expression, and subsequent PGE_2 and $PGF_{2\alpha}$ release in both fetal membranes and myometrial cells. This decrease in prostaglandin production could potentially detain the onset of uterine contraction and decrease the progression of cervical ripening, thus delaying the birth of the baby.

MMP-9 plays a role in the myometrium during parturition. During pregnancy the uterus is remodeled and enlarged by the addition of collagen to the myometrium, to accommodate the growing fetus, placenta, and amniotic fluid [44]. MMP-9 degrades this collagen during parturition, shrinking the uterus [44]. In addition, MMP-9 plays a major role in the degradation of the collagen matrix within fetal membranes, causing weakening, which, along with stretch forces, leads to membrane rupture [45]. Consistent with previous studies in nongestational tissues [46, 47], both luteolin and kaempferol attenuated IL-1β-induced MMP-9 activity in primary cells from amnion and myometrium. Of note, in myometrium, IL-1β did not increase MMP-9 gene expression, nor was there attenuation with luteolin or kaempferol. This may be explained by the fact that there are two different types of MMP-9 in myometrium, pro-MMP-9 and active MMP-9. The MMP-9 which is important in the context of parturition is active MMP-9, as this is the active form of the enzyme. qRT-PCR cannot distinguish between the two forms of MMP-9 and so hence gives the total MMP-9 expression, not just the active MMP-9 expression.

There is increasing evidence for the role of NF-κB and AP-1 in human term and preterm labour [6–9, 48]. Furthermore, mouse studies have been employed to demonstrate that by inhibiting NF-κB or AP-1, infection-induced PTB can be delayed [49, 50]. In nongestational tissues, luteolin and kaempferol are thought to exert their anti-inflammatory actions by inhibiting NF-κB [12–15] and AP-1 [14, 16, 17]. Similarly, in this study we show that luteolin and kaempferol

inhibited NF-κB DNA binding activity. In addition, both luteolin and kaempferol inhibited IL-1β-induced c-Jun expression, which is a nuclear protein that is part of the AP-1 transcription pathway. These findings suggest that, in human gestational tissues, luteolin and kaempferol may exert their inhibitory effects on proinflammatory cytokines, COX-2, prostaglandins, and MMP-9 via NF-κB. This is in agreement with previous studies, by our laboratory and others, that NF-κB and AP-1 regulate the transcription of prolabour mediators in human gestational tissues [6–9, 25].

There is emerging evidence for phytophenols as therapeutic agents for a number of pathological conditions including cancer [51]. They are readily available, inexpensive and have multitargeted potential [51]. However, their potential as therapeutics has also been heavily debated. They show low bioavailability and lose function due to metabolic processing when given via dietary supplementation [52]. If given at nutritionally relevant concentrations, extensive deglycosylation, glucuronidation, sulfation, and methylation reactions occur, mediated by a range of enzymes in the small intestine, liver, and colon. It has been shown that pharmacological doses that saturate metabolic pathways are required to obtain the free form of these phytophenols in the blood [52]. Whether luteolin or kaempferol can be used as therapeutics to prevent or delay PTB must first be addressed using experimental animal models of infection or inflammation-induced PTB. However, of promise is a recent study which has shown that kaempferol increases gestational length in a pregnant mouse model [53].

In addition to the anti-inflammatory actions, phytophenols possess a wide range of biological activities. For example, they have shown that both luteolin and kaempferol also possess antioxidant, antimicrobial, and anticancer activities [42, 43]. They have also been shown to have cardioprotective, antidiabetic, and neuroprotective effects. Luteolin has also shown antiallergic activity *in vitro* and *in vivo* [43].

In conclusion, in this study, we demonstrate that luteolin and kaempferol inhibit prolabour and proinflammatory mediators in human gestational tissues. Both luteolin and kaempferol have demonstrated anti-inflammatory properties in gestational tissues, by inhibiting NF-κB DNA binding activity, the AP-1 pathway, and their target genes. Given the central role of inflammation in provoking preterm labour, it is tempting to speculate that dietary phytophenols may be an effective, potential treatment or preventative for PTB. As a result of this research, further study is currently underway to determine effects of these phytophenols in an *in vivo* mouse model.

Conflict of Interest

There is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgments

The authors gratefully acknowledge the assistance of the Clinical Research Midwives Renee Grant, Asha Ferguson, Debra Jinks, Gabrielle Fleming, and the Obstetrics and Midwifery Staff of the Mercy Hospital for Women for their cooperation. During the period of this work, Dr. Martha Lappas was a recipient of a National Health and Medical Research Council (NHMRC) Career Development Award (Grant no. 1047025). The work described in this paper was funded by the Austin Hospital Medical Research Foundation (AMRF) and the Medical Research Foundation for Women and Babies.

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