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Effects of cytokine suppressive anti-inflammatory drugs (CSAIDs) on inflammatory activation in *ex-vivo* human and ovine fetal membranes.

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Short title: CSAIDs inhibit fetal membrane inflammation *ex vivo*.

ABSTRACT (250 words)

Intrauterine infection and inflammation are responsible for the majority of early (<32 weeks) spontaneous preterm births (sPTB). Anti-inflammatory agents, delivered intraamniotically together with antibiotics, may be an effective strategy for preventing PTB. In this study, the effects of four cytokine-suppressive anti-inflammatory drugs (CSAIDs: N-acetyl cysteine [NAC], SB239063, TPCA-1 and NEMO binding domain inhibitor [NBDI]) were assessed on human and ovine gestational membrane inflammation. Full-thickness membranes were collected from healthy, term, human placentas delivered by Caesarean section (n=5). Using a Transwell model they were stimulated *ex-vivo* with γ-irradiation-killed *E. coli* applied to the amniotic face. Membranes from near-term, ovine placentas were stimulated *in-utero* with either lipopolysaccharide (LPS), *Ureaplasma parvum* or saline control and subjected to explant culture. The effects of treatment with CSAIDs or vehicle [1% DMSO] on accumulation of PGE_2 and cytokines (human IL-6, IL-10, TNF-α; ovine IL-8) were assessed in conditioned media at various time points (3-20 h). In human membranes, the IKKβ inhibitor TPCA-1 (7 μM) and p38MAPK inhibitor SB239063 (20 μM) administered to the amniotic compartment were the most effective in inhibiting accumulation of cytokines and PGE_2 in the fetal compartment. NAC (10 mM) inhibited accumulation of PGE_2 and IL-10 only; NBDI (10 μM) had no significant effect. In addition to the fetal compartment, SB239063 also exerted consistent and significant inhibitory effects in the maternal compartment. TPCA-1 and SB239063 suppressed ovine IL-8 production, whilst all CSAIDs tested suppressed ovine PGE_2 production. These results support the further investigation of intraamniotically delivered CSAIDs for the prevention of inflammation-mediated preterm birth.

INTRODUCTION

Preterm birth (PTB) remains a persistent obstetric challenge associated with significantly increased risk of neonatal mortality as well as short-term and long-term morbidities (Goldenberg *et al.* 2008). The world-wide PTB rate is around 9.6%, with rates typically lower in developed countries (5-8%) and higher in developing nations (8-18%) (Lawn *et al.* 2010). In addition to its impact on individuals and their families, PTB carries a substantial economic cost, estimated to be ~$26 billion annually in USA in 2005 (Behrman & Stitch Butler 2006). Intrauterine infection and associated inflammation (most frequently diagnosed as presence of histological chorioamnionitis) have been identified as a cause of 30–40% of all spontaneous PTB. Up to 70% of very early spontaneous PTBs (<32 weeks gestation) are due to intrauterine infection-inflammation (Goldenberg *et al.* 2008).

Prophylactic antibiotic therapy has been extensively studied in the context of PTB prevention, with mixed results. While some studies have shown that administration of antibiotics (e.g. clindamycin) to high risk women early in pregnancy (<20 weeks gestation) can have positive benefits in terms of reduced PTB rates and improved perinatal outcomes (Lamont *et al.* 2011), the majority of trials have failed to show significant benefits (Barros *et al.*) and in some studies have even been shown to be harmful. The reasons for this are several fold, and include issues related to participant selection and antibiotic efficacy, tissue biodistribution and microbial resistance (Keelan 2011). In addition, bacteriocidal antibiotics cause bacterial lysis and release of endotoxins, further activating the innate immune system and promoting the release of prostaglandins which may actually stimulate the onset of labour (Dofferhoff *et al.* 1991, Hurley 1995, Holzheimer 2001).

We and others have proposed that a combined anti-inflammatory/antibiotic approach may be more effective than antibiotics alone in treating intrauterine infection-inflammation, prolonging pregnancy and preventing fetal exposure to an inflammatory environment (Keelan 2011, Grigsby *et al.*). Our present focus is on the intraamniotic administration of anti-inflammatory/antimicrobial agents in order to maximise therapeutic efficacy at the site of infection/inflammation, while minimising the risks of undesirable side-effects through the reduction of unintended maternal or fetal exposure. Most of the literature on anti-inflammatory drugs in PTB has focussed on non-steroidal anti-inflammatory drugs (NSAIDs) - prostaglandin synthesis inhibitors which have widespread applications but which have been associated with significant fetal side-effects (Kaplan *et al.* 1994, Nakhai-Pour *et al.* 2011). On the other hand, cytokine-suppressive anti-inflammatory drugs (CSAIDs) work by interfering with inflammatory signalling cascades and are therefore able to specifically block infection-mediated inflammation without some of the deleterious side-effects of NSAIDs (Lee *et al.* 1989) (Keelan 2011). CSAIDs have been shown to block inflammation in a variety of animal models of chronic inflammation (Underwood *et al.* 2000, Ward *et al.* 2001, Buhimschi *et al.* 2003, Jimi *et al.* 2004, di Meglio *et al.* 2005) as well as in human fetal membranes (Lappas *et al.* 2003, De Silva *et al.* 2010).

In the present study, we evaluated four CSAIDs that target two key signalling pathways known to be involved in inflammatory activation of fetal membranes: nuclear factor kappa B (NF-κB) (Lindstrom & Bennett 2005) and p38 mitogen-activated protein kinase (p38MAPK) (Lappas *et al.* 2007). The CSAIDs were: 1) NEMO binding domain inhibitor (NBDI); 2) N-acetyl cysteine (NAC); 3) TPCA-1 ([5-(*p*-fluorophenyl)-2-ureido] thiophene-3-carboxamide); and 4) SB239063 (trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-[2-methoxy)pyrim-idin-4-yl]imidazole). An intraamniotic model of drug delivery was employed, as this

approach allows for the targeting of gestational membranes and tissues (the key sites with respect to intraamniotic infection-driven PTB) with minimal risk of unintended maternal immune modulation.

This study aimed to assess and compare the anti-inflammatory efficacies of the four chosen CSAIDs on human and ovine gestational membranes using *ex-vivo* models of intraamniotic drug administration to assess their ability to inhibit inflammatory activation in both the amniotic and decidual faces of the gestational membranes.

MATERIALS AND METHODS

CSAIDs

Concentrations of CSAIDs were as follows: NAC (Enzo Life Sciences, New York, NY) 10 mM; NBDI (China Peptides, Shanghai, China) 10 µM; TPCA-1 (Merck Millipore, Darmstadt, Germany) 7 µM; SB239063 (Alexis Biochemicals, Lausen, Switzerland) 20 µM. Doses of CSAIDs were based on pilot studies or published data on *in-vitro* efficacy (Underwood *et al.* 2000, Barone *et al.* 2001, Ward *et al.* 2001, Ju *et al.* 2002, Lappas *et al.* 2003, Jimi *et al.* 2004, di Meglio *et al.* 2005, Tas *et al.* 2006, Shahin *et al.* 2009, De Silva *et al.* 2010, Grassia *et al.* 2010).

Human membrane Transwell model

Full thickness gestational membranes were collected from healthy, human, term placentas (38-40 weeks gestation) delivered by Caesarean section (n=5) with the approval of the local institutional Human Research Ethics Committee. Membranes were secured over 27 mm Transwell inserts (Corning Inc., Lindfield, Australia) and placed in 6-well culture plates containing serum-free culture media [DMEM/Ham’s Nutrient Mixture F-12, phenol red-free,

supplemented with 15 mM HEPES pH 7.3 (Sigma-Aldrich Co., St Louis, MO.), 0.5% endotoxin-/fatty acid-free BSA (Bovogen Biologicals Pty Ltd, East Keilor, Australia) and 4 μg/mL azithromycin (Pfizer, New York, NY.]. The maternal/decidual compartment contained 3 mL media, while the inner/amniotic compartment contained 2.5 mL. γ-irradiation killed *E. coli* (10 μg/mL) and fluorescent Spherobeads (40-60 nm, 0.1 mg/mL; Spherotech Inc, Lake Forrest, IL.) were added to the inner compartment, followed by CSAIDs in 1% DMSO or vehicle (1% DMSO control) at t=0 h. Membranes were incubated for 20 h at 37°C in 5% CO_2/95% air. Samples of conditioned media (100 μl) were taken from the fetal and maternal compartments at 0 h, 3 h, and 9 h and a final 1 mL sample was taken at 20 h. Structural integrity of the membranes was monitored by the passage of Spherobeads™ between inner/amniotic compartment and maternal/decidual compartments. Spherobead™ concentrations were measured in the fetal and maternal compartments by fluorescence using an FLx 800 plate fluorometer (BioTek Instruments Inc, Winooski, VT) at excitation 585/10 nm and emission 620/15 nm. Analysis of samples from both compartments of the Transwells showed that all membranes were intact with >99% of Spherobeads™ retained within the fetal compartments and no significant fluorescence detected in the maternal compartments of any of the Transwells.

Ovine membrane explant studies

Animal studies were performed on pregnant Merino sheep (*Ovis aries*) in Western Australia with the approval of the University of Western Australia's Animal Ethics Committee (RA/3/100/1098). The sheep in this study received intraamniotic injections at 117 ± 2 d gestational age (GA) of either saline (2 mL, n = 2), LPS (O55:B5; Sigma Aldrich; 10 mg in 2 mL saline, n = 4) or *U. parvum* serovar 3 (10^7 colour change units in 2 mL saline, n = 4) 7 days prior to delivery. All fetuses were surgically delivered at 124 ± 2 d GA (term = 150 d)

for necropsy. Fetal membranes were excised at this time and transported to the laboratory in media for explant culture. Explants were prepared from each set of membranes (8 mm discs), with 3 discs placed per well in 12-well plates and incubated in serum-free culture media at 37°C/5% CO_2/95% air. Treatment with the CSAIDs or vehicle (DMSO, 1%) was carried out for 14 h before the explants were removed, the media stored at -80°C for later analysis and the tissues air dried overnight and weighed for normalisation.

Measurement of cytokine and PGE_2 concentrations

Accumulation of cytokines (human IL-10, IL-6, TNF-α; ovine IL-8 [oIL-8]) and PGE_2 was measured in conditioned fetal and maternal media for the human Transwell study and from explant conditioned media for the ovine studies. Human IL-10 and TNF-α were measured by multiplex assay (Merck Millipore, Darmstadt, Germany) on a MAGPIX instrument (Luminex Corp, Austin, TX) as per the manufacturer's instructions. Human IL-6 was measured using an ELISA Development kit (PeproTech, New Jersey, USA) according to the recommended protocol. PGE_2 was measured by Prostaglandin E_2 EIA kit - monoclonal (Cayman Chemical Company, Michigan, USA) as per the manufacturer's instructions. Ovine IL-8 was measured by in-house ELISA calibrated against recombinant oIL-8 from Protein Express Inc. (Cincinnati, OH) using a mouse anti-sheep IL-8 monoclonal capture antibody (MCA1660: 5 µg/mL overnight) and a rabbit anti-sheep IL-8 polyclonal antibody (AHP425: 1:1000 2 h) from AbD Serotec (Raleigh, NC). Detection and quantitation involved an anti-rabbit IgG-HRPO conjugate (1:1000 1 h) and TMB substrate. The limits of detection of the IL-10, TNF-α, IL-6, PGE_2 and ovine IL-8 assays were <3.2, <3.2, 100, 7, and 33 pg/mL, respectively. Media samples were diluted 1:10 for the IL-6 assay, 1:5 for the PGE_2 assay, 1:2 for the IL-8 assay, and were undiluted for the IL-10 and TNF-α assays.

Statistical analysis

To adjust for variable baseline expression between membranes from different placentas, the concentrations of cytokines and PGE_2 within the conditioned media from each Transwell were expressed as a percentage of the sum of concentrations from all six Transwells from each set of membranes. The production data from the sheep explants were similarly normalised prior to statistical analysis. Data are shown as median ± inter-quartile range (IQR) or mean ± standard error of the mean (SEM). Unless stated otherwise, all statistical significance was assessed by one way analysis of variance (ANOVA) followed by Dunnett's *t-test* post-hoc analyses (Prism, GraphPad Software Inc., California, USA). Non-parametric data were log transformed prior to analysis. A *P*-value of <0.05 was considered significant. For analysis of basal and stimulated cytokine production rates in human membranes, significance was assessed by Wilcoxon matched pairs test, both at each individual time-point and overall.

RESULTS

Cytokine and prostaglandin production by stimulated human fetal membranes in the Transwell perfusion model

Figure 1 shows the baseline production of cytokines and PGE_2 over time (3, 9 and 20 h) in vehicle (DMSO) or *E. coli*-stimulated human Transwells. Basal PGE_2 accumulation in the fetal compartment increased modestly from 3 to 9 h, then declined at 20 h (**Figure 1A**); mean concentrations at 9 h were ~700 pg/mL. Basal PGE_2 levels were a little higher in the maternal compartment, peaking at approximately 1000 pg/mL at 9 h before declining by 20 h. With bacterial stimulation, however, levels in the fetal compartment rose markedly at 3 h to >1700 pg/mL and then declined thereafter, whereas in the maternal compartment no evidence of stimulation was observed. The effect of stimulation in the fetal compartment was significant

at 20 h (P<0.05) and overall time-points (P<0.001). TNF-α accumulation in the fetal compartment under basal conditions also peaked at the 9 h incubation period, reaching approximately 1300 pg/mL (**Figure 1B**). Similarly, maternal basal TNF-α concentrations also peaked at 9 h (~2900 pg/mL), then declined to ~1000 pg/mL at 20 h. With bacterial stimulation, concentrations of TNF-α in the fetal compartment were significantly (2-3 fold) elevated at 3 h and 20 h (P<0.05) (overall significance: P<0.001), with a significant stimulation also seen in the maternal compartment (P<0.05 overall). Fetal IL-10 levels were low or undetectable at 3 h, but rose to concentrations of approximately 300 pg/mL at 9 h before declining at 20 h (**Figure 1C**). Maternal basal IL-10 levels were significantly higher than fetal levels at 3 h (P<0.01) and peaked at 1100 pg/mL at 9 h, after which they progressively declined to 20 h. Stimulation with *E.coli* failed to increase IL-10 levels in either compartment.

Anti-inflammatory effects of CSAIDs on human fetal membranes

Treatment of *E.coli*-stimulated human gestational membranes at the amniotic face with NBDI had no significant effects on PGE_2 accumulation in either compartment, although at the 9 h time point median maternal PGE_2 levels were reduced by approximately 50%. Treatment with NAC resulted in a non-significant 60% reduction in PGE_2 accumulation relative to DMSO controls in the fetal compartment at 9 h, and a smaller (~35%) reduction in the maternal compartment at 20 h (**Figure 2A**); TPCA-1 resulted in significant (~70%; P<0.05) suppression of PGE_2 accumulation in the fetal (but not maternal) compartment at 3, 9, and 20 h post treatment **(Figure 2A**). SB239063 also significantly inhibited PGE_2 accumulation at all time points in the fetal compartment (80-85%; P<0.05), but unlike TPCA-1 it was also able to significantly reduce PGE_2 levels in the maternal compartment (approximately 70 and 87% at 9 and 20 h; P<0.05 and <0.001, respectively).

Neither NBDI nor NAC significantly affected TNF-α levels at any time point (**Figure 2B**). TNF-α accumulation in the fetal compartment was, however, markedly reduced by both TPCA-1 and SB239063, with significant reductions observed at 3 h ($P<0.01$), becoming more evident at 9 and 20 h ($P<0.001$). TPCA-1 significantly reduced maternal TNF-α accumulation by ~75% at 9 h ($P<0.05$). SB239063 was again the most effective anti-inflammatory agent in the maternal compartment with significant inhibitions of ~95% and ~62% seen at 9 and 20 h, respectively ($P<0.001$).

NBDI had no effect on IL-10 production in either compartment; however, IL-10 accumulation was inhibited by the other CSAIDs at the 9 and 20 h time points (**Figure 2C**). Within the fetal compartment, NAC, TPCA-1, and SB239063 all resulted in significant reductions in IL-10 production with effects at 20 h in the region of 85-92% ($P<0.001$). The same three CSAIDs reduced IL-10 accumulation in the maternal compartment, but the level of inhibition did not reach statistical significance due to large variability in the vehicle controls. The inhibitory effect of the anti-inflammatory agents tended to increase with time, although this trend was not statistically significant. The effect was most apparent for IL-10 (**Fig 2C**).

Inhibition of IL-6 accumulation was assessed at 20 h only, due to insufficient media at the earlier time points. Once more, NBDI failed to exert significant effects. There was again a trend towards inhibition by NAC and TPCA-1 in the fetal compartment, although the degree of inhibition was more modest than that seen for the other cytokines (38-62%) (**Figure 2D**). SB239063 was the most effective anti-inflammatory agent in both compartments and significantly inhibited IL-6 accumulation at the 20 h time-point ($P<0.05$).

Anti-inflammatory effects of CSAIDs on ovine fetal membrane explants

The efficacies of the four CSAIDs were evaluated in full thickness gestational membranes from near-term sheep. Explants were employed for the ovine studies as attempts to replicate the human Transwell study with ovine membranes were not successful. CSAID dosages and incubation times were based on results from the human studies. Samples were initially assayed for ovine IL-1β, IL-8, IL-10, TNF-α, MCP-1 and PGE_2; however, only concentrations of IL-8 and PGE_2 were above the detection limits of the assays employed and generated meaningful data.

Median PGE_2 concentration in media from full-thickness gestational membrane explants from saline treated sheep was 9.1 pg/mg tissue at 14 h, while median oIL-8 levels were 53.0 pg/mg tissue. Membranes from sheep stimulated with LPS or *U. Parvum* exhibited modest and variable increases in production of oIL-8 and PGE_2 that did not reach statistical significance compared to saline-treated controls. Therefore, the data from all three groups (n=9 sets of membranes) were analysed collectively.

In contrast to the human study, NBDI was as effective as the other CSAIDs at inhibiting PGE_2 accumulation in ovine gestational membranes. PGE_2 accumulation was significantly inhibited (65-71%; $P<0.01$) by all four CSAIDs compared to the DMSO vehicle treated explants (**Figure 3A**). However, NBDI and NAC had no effect on oIL-8 levels, and the effects of NAC treatment on oIL-8 levels was particularly variable. TPCA-1 significantly reduced oIL-8 accumulation by 80%; ($P<0.01$), while SB239063 significantly reduced oIL-8 levels by ~60% ($P<0.01$) (**Figure 3B**).

DISCUSSION

Preterm birth remains a major obstetric issue throughout the world and is associated with significant perinatal morbidity and mortality and life-long health and economic consequences. In 2010, 14.9 million preterm deliveries occurred worldwide, from which over 1 million infants died as a result of their prematurity (Blencowe *et al.* 2012). Despite decades of research on PTB aetiologies, few therapeutic options are available to women at risk of delivering preterm. Here, we have investigated the *ex-vivo* efficacy of a number of anti-inflammatory agents based on the hypothesis that intraamniotic CSAID administration can provide a pharmacological strategy for the prevention of infection/inflammation-mediated PTB. The CSAIDs selected for the present study were 1) NBDI, a cell permeable peptide that spans the NF-κB essential modifier (NEMO) binding domain (NBD) sequence (Madge & May 2009) which has been shown to block inflammation effectively in *in-vivo* animal models (Jimi *et al.* 2004, di Meglio *et al.* 2005, Tas *et al.* 2006, Grassia *et al.* 2010); 2) N-acetyl cysteine (NAC), a powerful antioxidant and free radical scavenger which has been shown in a randomised controlled trial to reduce the rate of preterm birth when taken orally in women with a history of preterm birth and in whom bacterial vaginosis has recently been treated (Shahin *et al.* 2009); 3) TPCA-1, a selective IKKβ inhibitor (Podolin *et al.* 2005, Kondo *et al.* 2008) that is effective at inhibiting inflammation *in-vitro* (Podolin *et al.* 2005, Sachse *et al.* 2011) and *in-vivo* (Birrell et al. 2006); 4) SB239063, a selective, potent and cell permeable p38MAPK inhibitor which has previously been shown to suppress inflammation *in-vivo* (Underwood *et al.* 2000, Barone *et al.* 2001, Ward *et al.* 2001, Ju *et al.* 2002). They were studied in a human Transwell system to model the structural characteristics of intact gestational membranes and allow the assessment of efficacy of intraamniotic anti-inflammatory drug delivery at both the maternal and fetal faces of the membranes. In parallel,

in-vivo stimulated ovine fetal membranes were also exposed to the CSAIDs to allow a comparison of efficacy between ovine and human tissues. DMSO vehicle was employed as previous studies in our laboratory have indicated that this solvent does not significantly alter inflammatory cytokine production by gestational tissues.

While all four CSAIDs showed some degree of efficacy in both models, two were clearly superior: the IKKβ inhibitor TCPA-1 and the p38MAPK inhibitor SB239063. At the concentrations employed, both of these compounds induced profound inhibitory effects on cytokine and prostaglandin accumulation in the fetal compartment of the Transwell model, with the MAPK inhibitor exerting more modest effects in the maternal compartment. The same degree of inhibition by these two CSAIDs was also seen in the ovine explant model, regardless of mode of stimulation. These findings support our hypothesis and provide rationale for the further investigation of these compounds in human gestational tissues derived from spontaneous preterm deliveries.

The central importance of NF-κB activation in the regulation of inflammatory gene expression is well recognised. We have previously shown that 5-7 μM TPCA-1 achieved ~90% suppression of pro-inflammatory cytokine production and blocks nuclear translocation of p65/RelA in LPS-stimulated choriodecidual cells (De Silva *et al.* 2010). Until now, no studies have examined the effect of TPCA-1 in full thickness gestational membranes although it has been shown to be an effective inhibitor of the NF-κB pathway in a variety of other inflammatory models (Podolin *et al.* 2005, Birrell *et al.* 2006, Kondo *et al.* 2008, Du *et al.* 2012). Interestingly, in the present study the actions of TPCA-1 were primarily restricted to the fetal (amniotic) compartment. This may reflect a lack of ability to penetrate the membrane barrier, restricting its actions to the amniotic epithelium, or may indicate that a

higher dose is required to more completely block the trans-membrane inflammatory signalling cascades. TPCA-1 also reduced PGE_2 and IL-8 production from ovine fetal membranes, confirming its effectiveness as an IKKβ inhibitor in the ovine species.

In contrast, the p38MAPK inhibitor SB239063 was much more effective at inhibiting cytokine and prostaglandin accumulation at the maternal face, suggesting that it is either considerably more membrane-permeable or has more profound effects on initial inflammatory signalling pathways. Its similar potency to TPCA-1 in the ovine explant model would argue against the latter hypothesis. To date, no studies have characterised the expression and activity patterns of MAPKs during inflammation in human gestational membranes, although MAPKs are known to respond to infectious stimuli and regulate the production of pro-inflammatory cytokines (Underwood *et al.* 2000, Barone *et al.* 2001, Ward *et al.* 2001, Ju *et al.* 2002, Shoji *et al.* 2007). Regardless, our results highlighted SB239063 as a potentially effective anti-inflammatory agent useful for preventing inflammation-driven PTB.

At the concentration used (10 μM) NBDI was unable to inhibit production of any of the measured inflammatory markers in either compartment of the human Transwell model. We had selected a dose of 10 μM of NBDI as this peptide has been successfully used at 0.1 - 1 μM in a study of injury induced inflammation in rats (Grassia *et al.* 2010). Surprisingly though, it was notably more effective in the ovine explants at suppressing PGE_2 accumulation. It remains to be determined whether a species difference in binding affinity or a relative insensitivity of the amnion membrane might explain these observations.

NAC, which exerts its effects through dampening of oxygen free radical reactions, has been shown to suppress NF-κB DNA binding activity in all three layers of gestational membranes at ≥10 mM (Lappas *et al.* 2003). In our study, NAC (10 mM) was not a particularly effective inhibitor of fetal membrane cytokine production, but did appear to reduce PGE_2 accumulation in the fetal side of the human Transwells. It also significantly reduced PGE_2 levels in conditioned media from sheep membrane explants. NAC can directly inhibit prostaglandin biosynthesis via inhibition of the production of PGH_2 by cyclooxygenases (De Flora *et al.* 2001), a reaction which involves a free radical step (Rouzer & Marnett 2009), so its effects on prostaglandin inhibition are consistent with expectations. These findings add some weight to the evidence that NAC might be an effective anti-inflammatory agent within the pregnant uterus (Lappas *et al.* 2003) and may be useful at preventing spontaneous PTB in some pregnancies (Shahin *et al.* 2009, Awad *et al.* 2011). The effectiveness of intraamniotic delivery of NAC *in-vivo* has not yet been explored.

The ovine membrane explant model used in this study employed membranes exposed *in-vivo* to either saline, LPS or *U. parvum*. This model has been developed over many years and is now extensively employed in obstetrics research (Kallapur *et al.* 2001, Moss *et al.* 2003, Moss *et al.* 2005). Unexpectedly, we did not observe a consistent and significant difference in PGE_2 or IL-8 production from control or stimulated membranes, although mean levels of both mediators were 2-3 fold higher in the stimulated membranes compared to controls. The inter-animal variability might have been due to regional differences in levels of activation of membranes. Due to the lack of significance, the data from all membranes were combined and hence we are unable to make conclusions regarding the efficacy of the CSAIDs with respect to different stimuli.

In conclusion, the results presented in this study identified TPCA-1 and SB239063 as CSAIDs of promise for pharmacological prevention of intraamniotic inflammation. Further *in-vivo* studies are justified to explore their ability to ameliorate the negative effects of intrauterine infection-driven inflammation. In combination with an effective antibiotic regimen, CSAIDs administered intraamniotically may have significant clinical benefits in treating pregnancies at high risk of spontaneous PTB due to intrauterine infection-inflammation.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could prejudice the impartiality of the research presented.

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399

400 **Figure legends**

401 **FIGURE 1**. Time-dependent changes in accumulation of **A:** PGE_2, **B:** TNF-α, **C:** IL-10 in

402 the conditioned media from the maternal and fetal compartments of human fetal membranes

403 in the Transwell model following exposure to vehicle (basal) or 10 µg/mL γ-irradiation killed

404 *E. coli* (stimulated) at the amniotic face. Data shown are concentration (pg/ml), mean ± SEM

405 (n=5 sets of membranes). **P<0.05 and ^P<0.001* basal *vs*. stimulated by Wilcoxon matched

406 pairs test.

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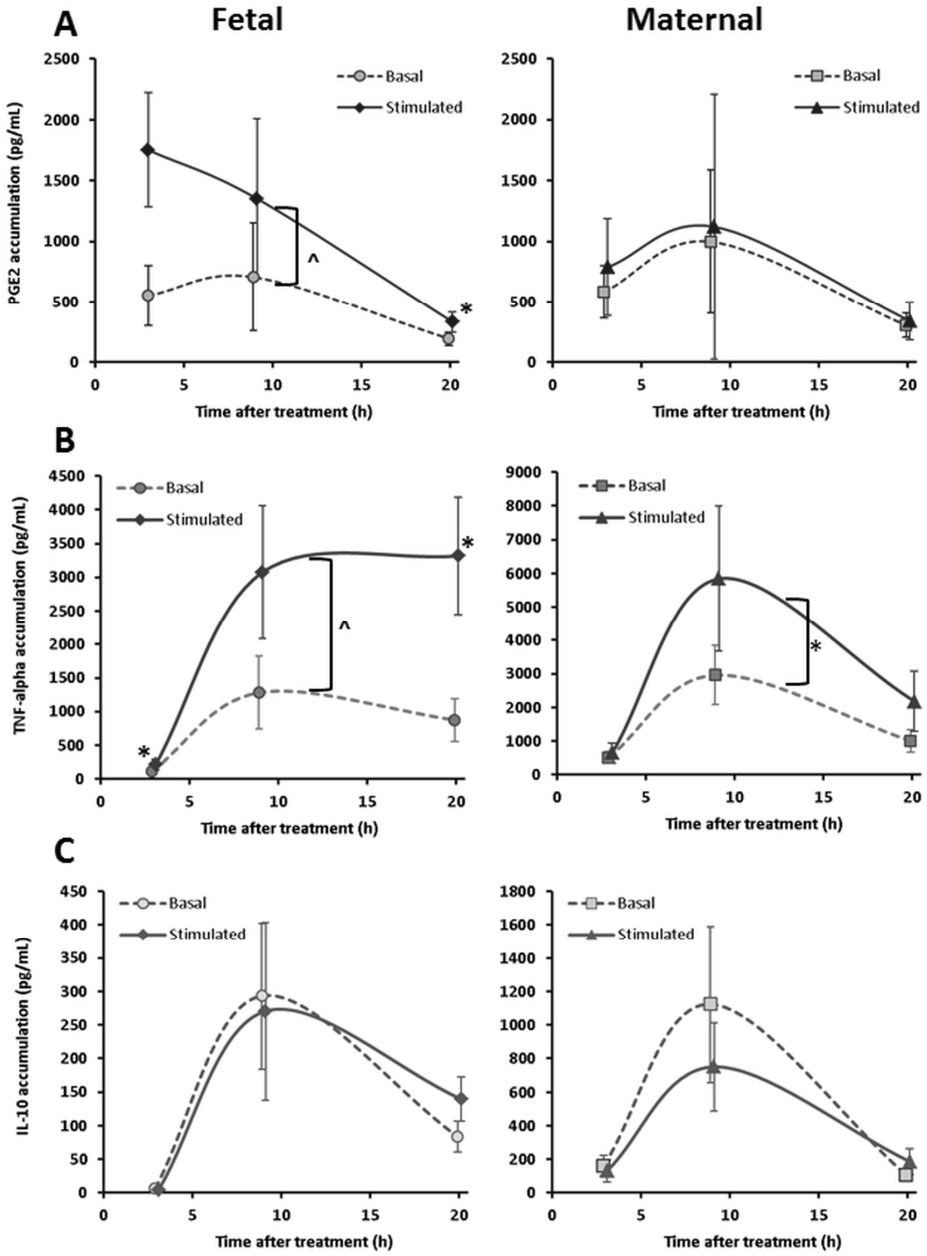

409 **FIGURE 2.** Efficacy of CSAIDs on *E. coli* stimulated **A:** PGE_2, **B:** TNF-α, **C:** IL-10 and **D**:
410 IL-6 production by human full thickness fetal membranes in an *ex-vivo* Transwell perfusion
411 model at 3 h, 9 h and 20 h culture. Data are median ± IQR from n=5 placentas, normalised as
412 a percentage of total analyte production per set of experiments. $^{*}P<0.05$, $^{\#}P<0.01$, $^{\wedge}P<0.001$
413 relative to vehicle (DMSO) control. Significance was assessed by two-tailed ANOVA after
414 log-transformation of data.
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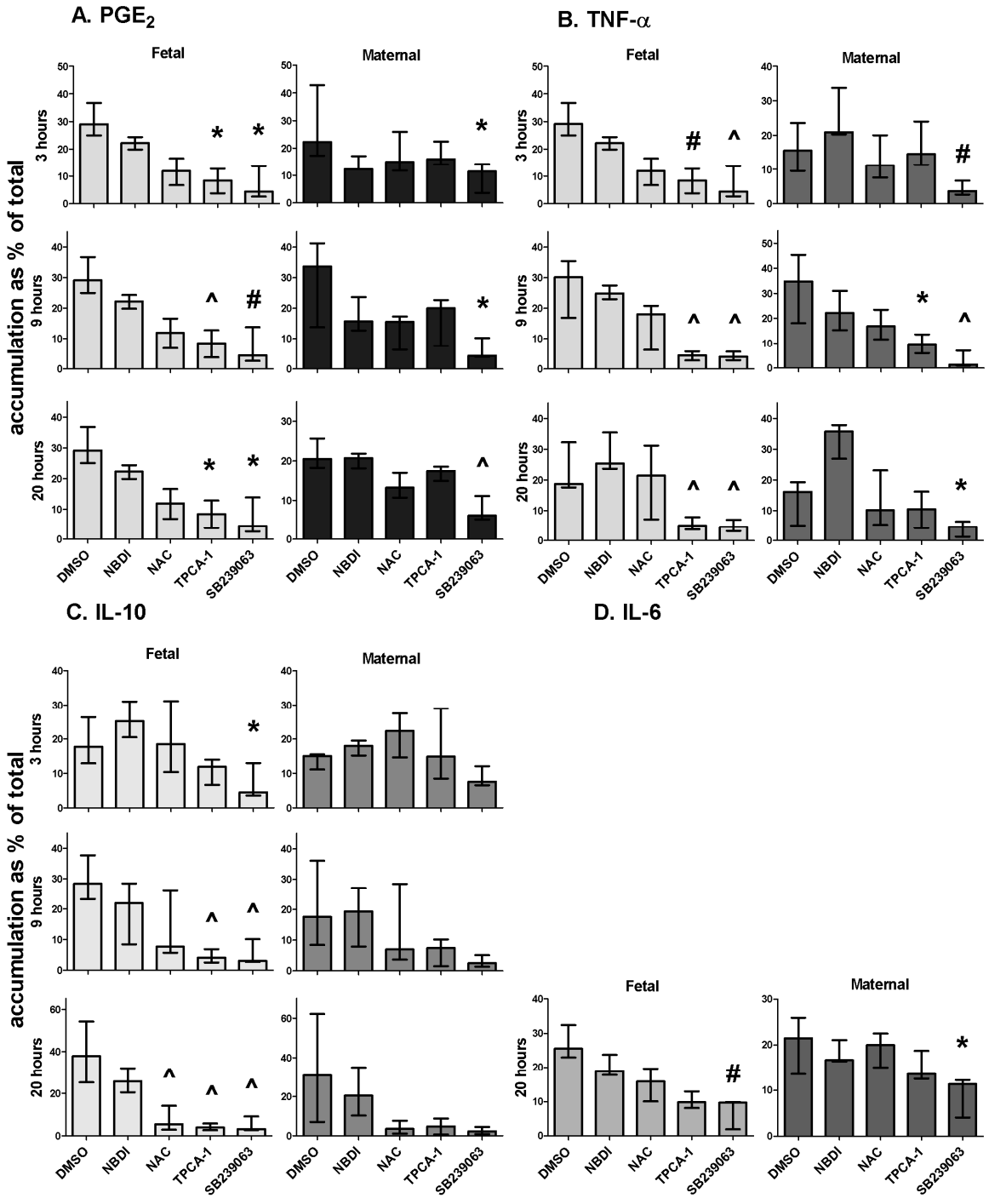

417 **FIGURE 3**. Effects of CSAIDs on **A:** PGE_2, and **B:** IL-8 accumulation in ovine fetal

418 membranes (n=9) over 14 h in explant culture. Data are expressed as a % of total

419 accumulation for each set of explants (median ± IQR). $^{**}P<0.01$ relative to vehicle control

420 (DMSO). Significance was assessed by two-tailed ANOVA after log-transformation of data.

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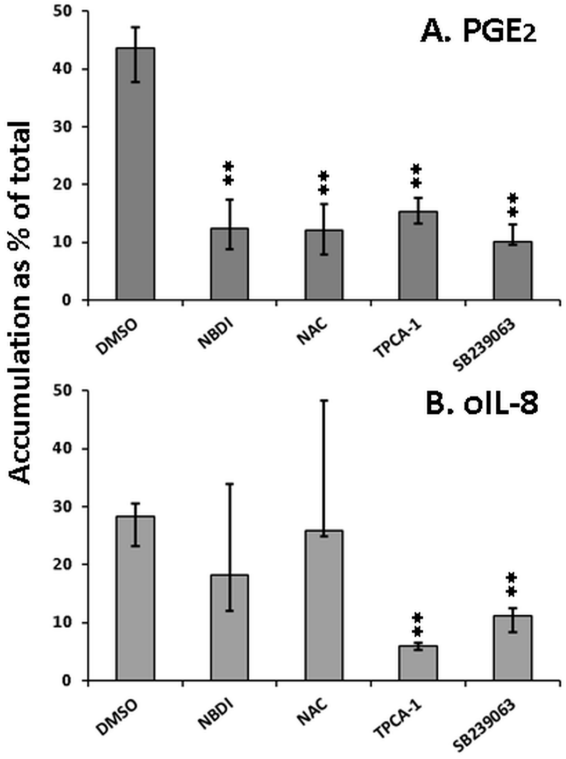

REFERENCES

Allport VC, Pieber D, Slater DM, Newton R, White JO & Bennett PR 2001 Human labour is associated with nuclear factor-kappaB activity which mediates cyclo-oxygenase-2 expression and is involved with the 'functional progesterone withdrawal'. *Mol Hum Reprod* **7** 581-586.

Awad N, Khatib N, Ginsberg Y, Weiner Z, Maravi N, Thaler I, Ross MG, Itsokovitz-Eldor J & Beloosesky R 2011 N-acetyl-cysteine (NAC) attenuates LPS-induced maternal and amniotic fluid oxidative stress and inflammatory responses in the preterm gestation. *Am J Obstet Gynecol* **204** 450 e415-420.

Barone FC, Irving EA, Ray AM, Lee JC, Kassis S, Kumar S, Badger AM, White RF, McVey MJ, Legos JJ, Erhardt JA, Nelson AH, Ohlstein EH, Hunter AJ, Ward K, Smith BR, Adams JL & Parsons AA 2001 SB 239063, a second-generation p38 mitogen-activated protein kinase inhibitor, reduces brain injury and neurological deficits in cerebral focal ischemia. *J Pharmacol Exp Ther* **296** 312-321.

Barros FC, Bhutta ZA, Batra M, Hansen TN, Victora CG & Rubens CE 2010 Global report on preterm birth and stillbirth (3 of 7): evidence for effectiveness of interventions. *BMC Pregnancy Childbirth* **10 Suppl 1** S3.

Behrman RA & Stitch Butler A 2006 *Preterm Birth: Causes, Consequences and Prevention*. Washington, D.C.: National Academies Press.

Birrell MA, Wong S, Hardaker EL, Catley MC, McCluskie K, Collins M, Haj-Yahia S & Belvisi MG 2006 IkappaB kinase-2-independent and -dependent inflammation in airway disease models: relevance of IKK-2 inhibition to the clinic. *Mol Pharmacol* **69** 1791-1800.

Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, Adler A, Vera Garcia C, Rohde S, Say L & Lawn JE 2012 National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet* **379** 2162-2172.

Buhimschi IA, Buhimschi CS & Weiner CP 2003 Protective effect of N-acetylcysteine against fetal death and preterm labor induced by maternal inflammation. *Am J Obstet Gynecol* **188** 203-208.

De Flora S, Izzotti A, D'Agostini F & Balansky RM 2001 Mechanisms of N-acetylcysteine in the prevention of DNA damage and cancer, with special reference to smoking-related end-points. *Carcinogenesis* **22** 999-1013.

De Silva D, Mitchell MD & Keelan JA 2010 Inhibition of choriodecidual cytokine production and inflammatory gene expression by selective I-kappaB kinase (IKK) inhibitors. *Br J Pharmacol* **160** 1808-1822.

di Meglio P, Ianaro A & Ghosh S 2005 Amelioration of acute inflammation by systemic administration of a cell-permeable peptide inhibitor of NF-kappaB activation. *Arthritis Rheum* **52** 951-958.

Dofferhoff AS, Nijland JH, de Vries-Hospers HG, Mulder PO, Weits J & Bom VJ 1991 Effects of different types and combinations of antimicrobial agents on endotoxin release from gram-negative bacteria: an in-vitro and in-vivo study. *Scand J Infect Dis* **23** 745-754.

Du Z, Whitt MA, Baumann J, Garner JM, Morton CL, Davidoff AM & Pfeffer LM 2012 Inhibition of type I interferon-mediated antiviral action in human glioma cells by the IKK inhibitors BMS-345541 and TPCA-1. *J Interferon Cytokine Res* **32** 368-377.

Goldenberg RL, Culhane JF, Iams JD & Romero R 2008 Epidemiology and causes of preterm birth. *Lancet* **371** 75-84.

Grassia G, Maddaluno M, Musilli C, De Stefano D, Carnuccio R, Di Lauro MV, Parratt CA, Kennedy S, Di Meglio P, Ianaro A, Maffia P, Parenti A & Ialenti A 2010 The I{kappa}B kinase inhibitor nuclear factor-{kappa}B essential modulator-binding domain peptide for inhibition of injury-induced neointimal formation. *Arterioscler Thromb Vasc Biol* **30** 2458-2466.

Grigsby PL, Novy MJ, Sadowsky DW, Morgan TK, Long M, Acosta E, Duffy LB & Waites KB 2012 Maternal azithromycin therapy for Ureaplasma intraamniotic infection delays preterm delivery and reduces fetal lung injury in a primate model. *Am J Obstet Gynecol* **207** 475 e471-475 e414.

Holzheimer RG 2001 Antibiotic induced endotoxin release and clinical sepsis: a review. *J Chemother* **13 Spec No 1** 159-172.

Hurley JC 1995 Antibiotic-induced release of endotoxin. A therapeutic paradox. *Drug Saf* **12** 183-195.

Jimi E, Aoki K, Saito H, D'Acquisto F, May MJ, Nakamura I, Sudo T, Kojima T, Okamoto F, Fukushima H, Okabe K, Ohya K & Ghosh S 2004 Selective inhibition of NF-kappa B blocks osteoclastogenesis and prevents inflammatory bone destruction in vivo. *Nat Med* **10** 617-624.

Ju H, Nerurkar S, Sauermelch CF, Olzinski AR, Mirabile R, Zimmerman D, Lee JC, Adams J, Sisko J, Berova M & Willette RN 2002 Sustained activation of p38 mitogen-activated protein kinase contributes to the vascular response to injury. *J Pharmacol Exp Ther* **301** 15-20.

Jung HS, Yoon BH, Jun JK, Kim M, Kim YA & Kim CJ 2005 Differential activation of mitogen activated protein kinases and nuclear factor-kappaB in lipopolysaccharide-treated term and preterm amnion cells. *Virchows Arch* **447** 45-52.

Kallapur SG, Kramer BW, Knox CL, Berry CA, Collins JJ, Kemp MW, Nitsos I, Polglase GR, Robinson J, Hillman NH, Newnham JP, Chougnet C & Jobe AH 2011 Chronic fetal exposure to Ureaplasma parvum suppresses innate immune responses in sheep. *J Immunol* **187** 2688-2695.

Kallapur SG, Willet KE, Jobe AH, Ikegami M & Bachurski CJ 2001 Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. *Am J Physiol Lung Cell Mol Physiol* **280** L527-536.

Kaplan BS, Restaino I, Raval DS, Gottlieb RP & Bernstein J 1994 Renal failure in the neonate associated with in utero exposure to non-steroidal anti-inflammatory agents. *Pediatr Nephrol* **8** 700-704.

Keelan JA 2011 Pharmacological inhibition of inflammatory pathways for the prevention of preterm birth. *J Reprod Immunol* **88** 176-184.

Kemp MW, Saito M, Newnham JP, Nitsos I, Okamura K & Kallapur SG 2010 Preterm birth, infection, and inflammation advances from the study of animal models. *Reprod Sci* **17** 619-628.

Kondo Y, Fukuda K, Adachi T & Nishida T 2008 Inhibition by a selective IkappaB kinase-2 inhibitor of interleukin-1-induced collagen degradation by corneal fibroblasts in three-dimensional culture. *Invest Ophthalmol Vis Sci* **49** 4850-4857.

Lamont RF, Nhan-Chang CL, Sobel JD, Workowski K, Conde-Agudelo A & Romero R 2011 Treatment of abnormal vaginal flora in early pregnancy with clindamycin for the prevention of spontaneous preterm birth: a systematic review and metaanalysis. *Am J Obstet Gynecol* **205** 177-190.

Lappas M, Permezel M & Rice GE 2003 N-Acetyl-cysteine inhibits phospholipid metabolism, proinflammatory cytokine release, protease activity, and nuclear factor-

kappaB deoxyribonucleic acid-binding activity in human fetal membranes in vitro. *J Clin Endocrinol Metab* **88** 1723-1729.

Lappas M, Permezel M & Rice GE 2007 Mitogen-activated protein kinase proteins regulate LPS-stimulated release of pro-inflammatory cytokines and prostaglandins from human gestational tissues. *Placenta* **28** 936-945.

Lawn JE, Gravett MG, Nunes TM, Rubens CE & Stanton C 2010 Global report on preterm birth and stillbirth (1 of 7): definitions, description of the burden and opportunities to improve data. *BMC Pregnancy Childbirth* **10 Suppl 1** S1.

Lee JC, Votta B, Griswold DE & Hanna N 1989 Inhibitory effect of SK&F 86002 on monocyte IL-1 production. *Agents Actions* **27** 280-281.

Lindstrom TM & Bennett PR 2005 The role of nuclear factor kappa B in human labour. *Reproduction* **130** 569-581.

Madge LA & May MJ 2009 Inhibiting proinflammatory NF-kappaB signaling using cell-penetrating NEMO binding domain peptides. *Methods Mol Biol* **512** 209-232.

Moss TJ, Nitsos I, Ikegami M, Jobe AH & Newnham JP 2005 Experimental intrauterine Ureaplasma infection in sheep. *Am J Obstet Gynecol* **192** 1179-1186.

Moss TJ, Nitsos I, Newnham JP, Ikegami M & Jobe AH 2003 Chorioamnionitis induced by subchorionic endotoxin infusion in sheep. *Am J Obstet Gynecol* **189** 1771-1776.

Nakhai-Pour HR, Broy P, Sheehy O & Berard A 2011 Use of nonaspirin nonsteroidal anti-inflammatory drugs during pregnancy and the risk of spontaneous abortion. *CMAJ* **183** 1713-1720.

Onderdonk AB, Delaney ML, DuBois AM, Allred EN & Leviton A 2008 Detection of bacteria in placental tissues obtained from extremely low gestational age neonates. *Am J Obstet Gynecol* **198** 110 e111-117.

Podolin PL, Callahan JF, Bolognese BJ, Li YH, Carlson K, Davis TG, Mellor GW, Evans C & Roshak AK 2005 Attenuation of murine collagen-induced arthritis by a novel, potent, selective small molecule inhibitor of IkappaB Kinase 2, TPCA-1 (2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide), occurs via reduction of proinflammatory cytokines and antigen-induced T cell Proliferation. *J Pharmacol Exp Ther* **312** 373-381.

Rouzer CA & Marnett LJ 2009 Cyclooxygenases: structural and functional insights. *J Lipid Res* **50 Suppl** S29-34.

Sachse F, Becker K, Basel TJ, Weiss D & Rudack C 2011 IKK-2 inhibitor TPCA-1 represses nasal epithelial inflammation in vitro. *Rhinology* **49** 168-173.

Shahin AY, Hassanin IM, Ismail AM, Kruessel JS & Hirchenhain J 2009 Effect of oral N-acetyl cysteine on recurrent preterm labor following treatment for bacterial vaginosis. *Int J Gynaecol Obstet* **104** 44-48.

Shoji T, Yoshida S, Mitsunari M, Miyake N, Tsukihara S, Iwabe T, Harada T & Terakawa N 2007 Involvement of p38 MAP kinase in lipopolysaccharide-induced production of pro- and anti-inflammatory cytokines and prostaglandin E(2) in human choriodecidua. *J Reprod Immunol* **75** 82-90.

Sooranna SR, Engineer N, Loudon JA, Terzidou V, Bennett PR & Johnson MR 2005 The mitogen-activated protein kinase dependent expression of prostaglandin H synthase-2 and interleukin-8 messenger ribonucleic acid by myometrial cells: the differential effect of stretch and interleukin-1{beta}. *J Clin Endocrinol Metab* **90** 3517-3527.

Tas SW, Vervoordeldonk MJ, Hajji N, May MJ, Ghosh S & Tak PP 2006 Local treatment with the selective IkappaB kinase beta inhibitor NEMO-binding domain peptide ameliorates synovial inflammation. *Arthritis Res Ther* **8** R86.

Triantafilou M, De Glanville B, Aboklaish AF, Spiller OB, Kotecha S & Triantafilou K 2013 Synergic activation of toll-like receptor (TLR) 2/6 and 9 in response to ureaplasma parvum & urealyticum in human amniotic epithelial cells. *PLoS One* **8** e61199.

Underwood DC, Osborn RR, Kotzer CJ, Adams JL, Lee JC, Webb EF, Carpenter DC, Bochnowicz S, Thomas HC, Hay DW & Griswold DE 2000 SB 239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. *J Pharmacol Exp Ther* **293** 281-288.

Ward KW, Proksch JW, Azzarano LM, Salyers KL, McSurdy-Freed JE, Molnar TM, Levy MA & Smith BR 2001 SB-239063, a potent and selective inhibitor of p38 map kinase: preclinical pharmacokinetics and species-specific reversible isomerization. *Pharm Res* **18** 1336-1344.

Yao S, Luo ZZ, Li D & Zhou CJ 2008 [Study of the role of nuclear factor-kappa B in preterm birth with subclinical chorioamnionitis]. *Zhonghua Fu Chan Ke Za Zhi* **43** 670-674.