Intraamniotic pharmacological blockade of inflammatory signalling pathways in an ovine chorioamnionitis model

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Abstract

Intrauterine inflammation (IUI) associated with infection is the major cause of preterm birth (PTB) at less than 32 weeks’ gestation and accounts for approximately 40% of all spontaneous PTBs. Pharmacological strategies to prevent PTB and improve fetal outcomes will likely require both antimicrobial and anti-inflammatory therapies. Here we investigated the effects of two cytokine-suppressive anti-inflammatory drugs (CSAIDs), compounds that specifically target inflammatory signalling pathways, in an ovine model of lipopolysaccharide (LPS)-induced chorioamnionitis. Chronically catheterised ewes at 116 days gestation (n=7/group) received an intraamniotic (IA) bolus of LPS (10 mg) plus vehicle or CSAIDS: TPCA-1 (1.2 mg/kg fetal weight) or 5z-7-oxozeaenol (OxZnl; 0.4 mg/kg fetal weight); controls received vehicle (DMSO). Amniotic fluid (AF), fetal and maternal blood samples were taken 0, 2, 6, 12, 24 and 48 h; tissues were taken at autopsy (48 h). Administration of TPCA-1 or OxZnl abrogated the stimulatory effects of LPS (p<0.01 vs. vehicle control) on production of PGE$_2$ in AF, with lesser (non-significant) effects on IL-6 production. Fetal membrane polymorphonuclear cell infiltration score was significantly higher in LPS vs. vehicle control animals (p<0.01), and this difference was absent with TPCA-1 and OxZnl treatment. LPS-induced systemic fetal inflammation was highly variable, with no significant effects of CSAIDs observed. Lung inflammation was evident with LPS exposure, but unaffected by CSAID treatment. We have shown in a large animal model that IA administration of a single dose of CSAIDs can suppress LPS-induced IA inflammatory responses, while fetal effects were minimal. Further development and investigation of these compounds in infectious models is warranted.

Key words: Cytokine-suppressive anti-inflammatory drugs/ Intrauterine infection / Preterm birth/ TPCA-1/ 5z-7-oxozeaenol
Introduction

Preterm birth (PTB), defined as delivery less than 37 weeks of gestation, accounts for approximately 11% of all live births worldwide (Blencowe et al., 2013) and is associated with 70% of all neonatal mortality and morbidity (Goldenberg et al., 2008). The morbidity and mortality associated with PTB incurs significant human and financial costs (Petrou, 2005); the annual societal costs associated with PTB in the USA was estimated to be $26.2 billion in 2005 (Institute of Medicine (US) Committee on Understanding Premature Birth and Assuring Healthy Outcomes, 2007). Despite years of research into the mechanisms responsible for activation of preterm labour (PTL), and the introduction of medical interventions to prevent PTB, rates have continued to increase in developed countries (Blencowe et al., 2013).

Intrauterine inflammation (IUI) associated with infection is the major cause of early PTB (less than 32 weeks’ gestation) and accounts for approximately 40% of all spontaneous PTBs (DiGiulio et al., 2010, Kim et al., 2009, Romero et al., 2001). Activation of pathogen recognition receptors by bacteria, most commonly originating and ascending from the vagina (DiGiulio, 2012, DiGiulio et al., 2010, Jones et al., 2009), initiates a cascade of pro-inflammatory events, resulting in histologic chorioamnionitis and elevated amniotic fluid (AF) concentrations of pro-inflammatory cytokines, chemokines and prostaglandins (Agrawal et al., 2012, Elovitz et al., 2003, Romero et al., 2001). These events trigger uterine contractions and initiation of PTL. In the most severe cases, IUI can lead to fetal inflammatory response syndrome (FIRS) (reviewed in (Gotsch et al., 2007)) characterised by increased fetal expression of chemokines and cytokines and damage to the cardiovascular,
pulmonary, hepatic, gastrointestinal and neurological systems (Fawke, 2007, Jobe, 2012, Lahra et al., 2009, Shatrov et al., 2010, Viscardi et al., 2004).

The administration of antibiotics to women for the treatment or prevention of preterm labour has been shown to have little effect on reducing rates of preterm birth or neonatal morbidity and mortality (Joergensen et al., 2014a, Kenyon et al., 2008a, 2008b, Kenyon et al., 2001a, 2001b, Simcox et al., 2007). As maternal and fetal inflammation associated with IUI is usually well established at presentation with PTL, effective treatment is required to both combat the infection and reduce the associated inflammatory response (Keelan, 2011, Rinaldi et al., 2011). In an important proof-of-principle study, co-treatment of a high risk cohort of women with bacterial vaginosis with N-acetyl cysteine (NAC), a non-specific inhibitor of inflammatory signalling pathways, was shown to reduce the incidence of PTB, increase birth weight and reduce neonatal morbidity/mortality over and above the effects of 17-hydroxyprogesterone caproate alone (Shahin et al., 2009). However, NAC therapy has not progressed into mainstream clinical use, likely in part because it is poorly tolerated (Shahin et al., 2009) and its efficacy has yet to be confirmed in other studies.

Non-steroidal anti-inflammatory drugs (NSAIDs) were once commonly prescribed in pregnancy to treat fever, pain and inflammation, but their use has declined due to adverse effect profiles (Kaplan et al., 1994, Kaplan et al., 2013, Nakhai-Pour et al., 2011). Cytokine-suppressive anti-inflammatory drugs (CSAIDs) are a novel category of anti-inflammatory drugs that specifically target inflammatory signalling pathways (e.g. NF-κB or MAPK pathways), without the adverse and/or non-specific effects of NSAIDs (Griswold et al., 1993, Keelan, 2011). We recently compared the anti-inflammatory effects of NAC and three CSAIDs on stimulated human and ovine gestational membranes and found two drugs were
effective at suppressing cytokine and prostaglandin production in vitro (Stinson et al., 2014): an IKKβ inhibitor TPCA-1 (5-[p-fluorophenyl]-2-ureido] thiophene-3-carboxamide) (De Silva et al., 2010) and a MAPK inhibitor SB239063 (trans-1-[4-hydroxy-cyclohexyl]-4-[4-fluorophenyl]-5-[2-[methoxy]pyrim-idin-4-yl] imidazole) (Barone et al., 2001, Lappas et al., 2007). These data corroborated a much earlier study which demonstrated SKF86002, a CSAID prototype, inhibited levels of IL-1β protein, COX-2 mRNA expression and PGE2 production by full-thickness human gestational membranes stimulated in vitro with LPS (Sullivan et al., 2002). While the in vitro evidence suggests further investigation of CSAIDs for the prevention of inflammation-mediated PTB is warranted, it is unknown to what extent NF-κB/MAPK inhibitors would be safe and effective in vivo in terms of preventing preterm labour and/or treating FIRS.

The size and the gestational length (approximately 147 days) of sheep enables longitudinal pregnancy studies including non-invasive amniotic fluid sampling under ultrasound guidance, and surgical interventions such as long-term catheterization (reviewed in (Kemp et al., 2010). Intraamniotic LPS administration in sheep results in chorioamnionitis, elevated proinflammatory cytokine expression in the amnion/chorion, increased cytokine and prostaglandin levels in the amniotic fluid and fetal lung inflammation (Kallapur et al., 2001) mimicking that observed in the human condition and making it a good experimental model for targeting inflammatory responses in pregnancy. Although intraamniotic injection with LPS or live microorganisms does not induce preterm labour in sheep (Moss et al., 2005), this sheep model of IUI is ideal for pharmacological intervention studies, as it enables sustained longitudinal assessments without the complication imposed by premature labour and delivery. The present study, therefore, aimed to assess in an ovine model of LPS-induced chorioamnionitis the IA anti-inflammatory efficacies of two CSAIDs: TPCA-1 and 5z-7-
oxozeaenol (OxZnl), a selective fungal inhibitor of TAK1 (transforming growth factor beta-activated kinase 1) kinase and ATPase activity (Wu et al., 2013). We employed intraamniotic (IA) CSAID delivery to allow localised targeting of the gestational membranes and the fetus (the key sites of IA infection-driven inflammation) and minimise the risk of unintended maternal immune modulation. We hypothesised that IA-administered CSAIDs would be inhibit LPS-induced inflammation within the amniotic cavity and gestational membranes, with lesser or negligible effects on the fetus and mother.

**Methods**

**Animals, surgical procedures and CSAID administration**

All experimental procedures were approved by the Animal Ethics Committee of The University of Western Australia (RA/3/100/1203). Animal management, anaesthesia, surgical catheterisation, and recovery have all been previously described (Kemp et al., 2013). The experimental model is summarised diagrammatically in Figure 1). Surgery with catheterisation was performed on day 110 of gestation. At 116 days of gestation (equivalent to approx. 32 weeks of human gestation), three groups of chronically catheterized pregnant ewes received an intraamniotic (IA) bolus of 10 mg LPS from *E.coli* O55:B5 (LPS; L2880, Sigma Aldrich, St. Louis, MO) in 2 ml saline. Inflammatory effects of 48 h IA 10 mg LPS stimulation have been previously described (Kallapur et al., 2007, Saito et al., 2014). A fourth group of unstimulated animals received IA 2 ml saline (vehicle controls). One LPS stimulated group received IA 2.8 mg TPCA-1 (Merck Millipore, Darmstadt, Germany) in 1 ml dimethyl sulfoxide vehicle (DMSO; D8418, Sigma Aldrich); the second LPS stimulated group received IA 0.875 mg OxZnl (BioAustralis Fine Chemicals, Smithfield, Australia) in 1 ml DMSO, and the third LPS stimulated group received IA 1 ml DMSO. The unstimulated
group (vehicle control) also received IA 1 ml DMSO. TPCA-1 was administered IA to yield an approximate concentration of 20 μM in AF based on an approximate volume of 500 ml at 115 days sheep gestation (equivalent to 30 weeks’ human gestation) (Sandlin et al., 2014, Tomoda et al., 1985). OxZnl was administered to yield an approximate concentration of 5 μM in AF. The target doses were chosen based on concentration-response data from our preliminary and published fetal membrane studies; these studies indicated that concentrations of 20 μM TPCA-1 and 5 μM OxZnl are close to maximally effective in suppressing cytokine production without exerting toxicity (De Silva et al., 2010, Stinson et al., 2014). Maternal and fetal arterial blood and AF (two sets of 1 ml samples) were collected into heparinized tubes 30 min before administration of the LPS and CSAIDs. Samples were then also taken at 2, 6, 12, 24, and 48 h post-administration, by which time the effects of the CSAIDs were expected to have plateaued. Fetal arterial pH, PO$_2$, PCO$_2$, HCO$_3^-$ and base excess (BE) were measured with a Rapid Lab 1265 blood gas analyser (Siemens, Germany). Plasma and AF samples were stored at −80°C for analysis.

Haematology and liver function tests

Fetal arterial cord blood samples for liver function tests (AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; GLDH, glutamate dehydrogenase; ALB, albumin; TB, total bilirubin) were collected in a 10 mL SST® clot-activating Vacutainer® (BD, Franklin Lakes, NJ). Complete blood cell counts and differential analyses were performed by VetPath Laboratory Services (Perth, Western Australia) using an automated Coulter counter adapted for ovine specimens.
Measurement of cytokine and PGE$_2$ concentrations

Ovine IL-6 and IL-8 protein concentrations in AF and maternal and fetal plasma were determined by ELISA as previously described (Saito et al., 2014). Samples were analysed undiluted for IL-6 and at 1:50 dilution for IL-8. The top standard for IL-6 was 8000 pg/ml with a lower limit of detection of 150 pg/ml and average intra-assay coefficient of variability (CV) of 8%. The top standard for IL-8 was 8000 pg/ml with a lower limit of detection of 25 pg/ml and CV of 10%. Quantification of PGE$_2$ in AF was performed using a prostaglandin E$_2$ EIA Kit – monoclonal (Cayman Chemical Company, Ann Arbor, MI, USA) with unstimulated samples analysed at 1:10 dilution, LPS-stimulated samples at 1:250 dilution and LPS/CSAID samples at 1:20 dilution. The assay range PGE$_2$ was 10-1000 pg/ml with a CV of 9%. Samples falling below the lower limits of detection were assigned the concentration equivalent to the lower limit detected for statistical analysis.

Relative Quantification of mRNA Expression

Total RNA was extracted from fetal tissue using TRIzol (15596-018; Life Technologies, Carlsbad, CA.) and treated with Turbo DNase (AM2238; Life Technologies) to remove any residual DNA as previously described (Saito et al., 2014). RNA yields from fetal tissues were normalized to 25 ng/μl using nuclease-free water (AM9937; Life Technologies). Primers and hydrolysis probes specific for ovine IL-1β, IL-6, IL-8, TNF-α, MCP2, hepcidin, serum amyloid A3 (SAA) protein and C-reactive protein (CRP) and quantitative PCR conditions are all as previously described (Saito et al., 2014). The Cq values were normalized to 18s rRNA and expressed relative to pooled control values. There were no significant differences in the Cq values for 18s rRNA between the treatment groups in fetal liver (p=0.449; mean Cq ± SD for vehicle control, LPS/vehicle, LPS/OxZnl and LPS/TPCA-1 groups were 15.05 ± 0.17, 14.76 ± 0.25, 15.15 ± 0.10 and 15.10 ± 0.24, respectively) or fetal lung (p=0.868; mean Cq ±
SD for the four groups = 14.82 ± 0.10, 14.81 ± 0.03, 14.81 ± 0.08, 14.92 ± 0.05, respectively).

Fetal lung and membrane histology
Sections (5 µm thick) from formalin-fixed paraffin-embedded amnio-chorion and fetal lung (right upper lobe) were stained with haematoxylin and eosin (H&E). Stained sections were imaged using an Aperio ScanScope XT (Leica Biosystems, Nussloch, Germany) with 40× objective and imaged at 10× magnification using ImageScope version 11.2.0.780 (Aperio Technologies Inc. Leica Biosystems, Nussloch, Germany). Five random fields were assessed at low-power magnification and scored as previously described (Kemp et al., 2014). The scorer was blind to the group identity of the sections.

For fetal lung, scores were as follows:
0, normal
1, few airspace inflammatory cells and no consolidation
2, foci of airspace inflammatory cells with minimal microconsolidation
3, foci of airspace inflammatory cells with microconsolidation
4: extensive airspace inflammatory cells and consolidation

For chorion:
0, no chorioamnionitis
1, few polymorphonuclear (PMN) cells in subchorionic fibrin and/or membrane trophoblast layer (stage 1, grade 1 chorioamnionitis)
2, isolated foci or bands of PMN cells in subchorionic fibrin and/or membrane trophoblast layer (stage 1, grade 2 chorioamnionitis)
3, foci or continuous bands of PMN cells extending into amnion (stage 2, grade 2 chorioamnionitis)

4, confluent PMN cells, amniocyte necrosis and/or amnion basement membrane thickening (stage 3, grade 2 chorioamnionitis)

Statistical analysis

All data were analysed using GraphPad Prism version 6.04 for Windows (GraphPad Software, La Jolla, California, USA). Fetal measurements at delivery (birth weight, arterial cord blood gases and liver enzymes) are presented as mean ± SD and compared by one-way ANOVA with post-hoc Tukey’s multiple comparison tests employing an α-value of 0.05. Data representing concentrations of cytokines and PGE2 in AF and plasma are shown as box plots with median and whiskers representing 5th - 95th percentiles. Treatment effects over time were determined by ordinary two-way ANOVA (no matching) employing an α-value of 0.05. Treatment effects at individual time points were determined by Kruskal-Wallis tests with post-hoc Dunn’s multiple comparisons tests with an α-value of 0.05. Comparisons between unstimulated controls and combined LPS-stimulated groups were made using unpaired t-tests for normally distributed data and Mann-Whitney tests for non-parametric data with an α-value of 0.05.

Results

Final treatment group sample sizes

Two animals were found to have lost their pregnancies at the time of surgery (one vehicle control animal and one LPS/OxZnl animal) and were eliminated from the study. One fetus (LPS/vehicle) with apparent intrauterine growth restriction was eliminated from the study
(birth weight at delivery 1.3 kg). The fetal loss rate for catheterised animals was 11% (3/28 animals). Two fetuses died following administration of LPS and pharmacological agents (one LPS/TPCA-1 and one LPS/OxZnl). In both cases the fetuses were anatomically normal, with no signs of infection and all surgical installations intact; these animals were excluded from analyses. One fetus (LPS/OxZnl) was found dead mid-sampling, likely from exsanguination as a result of a torn fetal carotid catheter, and was excluded from analyses. None of these deaths appeared to be specifically attributed to the CSAID treatments. A final sample size of N=6 vehicle control, N=6 LPS/vehicle, N=4 LPS/OxZnl and N=6 LPS/TPCA-1 was available for study.

Effect of CSAIDS on intraamniotic inflammation

Intraamniotic administration of LPS (LPS/vehicle) stimulated PGE$_2$ production in AF (treatment factor p=0.0002 by two-way ANOVA; Figure 2). PGE$_2$ levels in AF were significantly increased at each time point in LPS/vehicle animals compared to unstimulated vehicle controls (p<0.05 by Kruskal-Wallis test). Co-administration of TPCA-1 or OxZnl abrogated the stimulatory effects of LPS on production of PGE$_2$, resulting in the loss of significance compared to vehicle control. Intraamniotic IL-6 concentrations were increased with LPS stimulation (treatment factor p=0.029). Whilst there was a trend towards reduced IL-6 levels with CSAID treatments, particularly prior to 12 h, this was not statistically significant at individual time points (Figure 2). Intraamniotic IL-8 production increased marginally over time in response to LPS stimulation (time factor p=0.002 and treatment factor p=0.072), with a statistically significant increase in IL-8 concentrations observed at 24 h with LPS stimulation (p<0.05 vs. vehicle control). Although median IL-8 levels appeared lower in the CSAID groups, particularly at early time points, the variability was considerable and differences were not statistically significant.
Analysis of H&E stained gestational membranes from LPS-stimulated animals demonstrated significant infiltration of PMN cells compared to vehicle controls (p=0.0007 by t-test for combined LPS animals vs. vehicle control; Figure 3). PMN infiltration score in the LPS/vehicle group was significantly increased vs. vehicle control (p<0.01). This difference was abolished with TPCA-1 and OxZnl treatments.

**Effects of CSAIDs on the preterm fetus**

Birth weight (g) was similar across the four groups (Table I). Fetal arterial cord total white blood cell numbers, lymphocyte numbers and monocyte numbers were comparable across groups at 48 h. The number of neutrophils was reduced in fetal arterial cord blood of all LPS-stimulated groups, although this difference only reached significance for the LPS/vehicle and LPS/TPCA-1 groups (p<0.01 vs. vehicle control). There were no significant differences in fetal arterial cord blood pH, pCO₂, HCO₃⁻, or BE(B) between groups. pO₂ was increased in the LPS/OxZnl group (p<0.05) vs. vehicle control. Fetal liver function tests revealed a marginal reduction in albumin concentration (p<0.05) in LPS/OxZnl animals compared to vehicle controls (Table II). Concentrations of liver enzymes (AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; GLDH, glutamate dehydrogenase) and total bilirubin were not different between groups.

**Effect of CSAIDs on maternal and fetal inflammation**

Maternal plasma concentrations of IL-8 were increased in LPS-stimulated animals (treatment factor p=0.027), particularly in the first 12 h. No significant inhibitory effect of either CSAID treatment was observed (Figure 4A). A significant increase in maternal circulating IL-6 was evident in the LPS/vehicle group at 6-12 h (treatment factor p=0.0005). This effect was
abrogated in animals treated with either TPCA-1 or OxZnl, although levels were not significantly lower when compared to the LPS/vehicle group.

Circulating fetal IL-6 and IL-8 concentrations were slower to respond to LPS stimulation and were only increased after 48 h for IL-8 (time factor p=0.010). IL-6 concentrations tended to be highest in the LPS/vehicle group at 48 h, but were highly variable and the differences were not statistically significant. IL-6 levels in the fetal circulation of CSAID treated animals were low or non-detectable and not significantly different from LPS/vehicle (Figure 4B).

Analysis of H&E stained fetal lung (right upper lobe) demonstrated significant infiltration of PMN cells in LPS animals (combined groups) compared to vehicle controls (p=0.0004 by t-test; Figure 5). PMN infiltration scores were significantly increased above vehicle control for LPS/vehicle (p<0.05) and LPS/TPCA-1 (p<0.01). OxZnl treatment inhibited this response.

The relative mRNA expression levels of selected cytokines and chemokines in fetal lung samples were determined at delivery (48 h). IA LPS stimulation (all groups) increased fetal lung mRNA expression of IL-1β (~20-fold; p=0.001, Mann Whitney), IL-6 (~4-fold; p=0.003, Mann Whitney), IL-8 (~80-fold; p=0.0001, Mann Whitney), TNF-α (~6-fold; p=0.013, t-test) and MCP-2 (~25-fold; p=0.005, Mann Whitney) compared to vehicle controls (Figure 6). However, when treatment groups were compared individually to vehicle control, only LPS/TPCA-1 group had increased cytokine/chemokine mRNA expression levels (p<0.05). There was no evidence for inhibition of mRNA expression by CSAID treatments at the 48 h time point.
Modest increases in acute-phase protein mRNA expression were detected at delivery in fetal liver of LPS stimulated animals vs. vehicle controls (Figure 7). Increases of ~2-fold for CRP (p=0.057, t-test) and Hepcidin (p=0.027, t-test) were observed, while a much more robust increase of ~180-fold was detected for SAA (p=0.0003, Mann Whitney). Only SAA expression was significantly increased in individual LPS-stimulated groups (LPS/TPCA-1 and LPS/OxZnl) vs. vehicle control (p<0.05). There was no evidence for inhibition of mRNA expression by CSAID treatment at the 48 h time point.

**Discussion**

We have shown here, using a well-validated large animal model, evidence to support our hypothesis that intraamniotic administration of a single dose of a CSAID can suppress intraamniotic inflammation in response to a microbially derived stimulus, LPS. Our data also suggest that IA administration of a CSAID has the potential to exert a beneficial effect on fetal inflammation with minimal effects on maternal circulating cytokine levels. Importantly, single dose treatment with the selected CSAIDs, TPCA-1 and OxZnl, appeared to have only minimal effects on fetal blood gas parameters, WBC counts and liver function. Hence, the administration of CSAIDS appears to be free of acute fetal adverse-effects, although it should be noted that their effects in the absence of LPS exposure were not evaluated in this experimental design.

Despite the knowledge of the importance of inflammation as a cause of PTB, anti-inflammatory modalities for the treatment or prevention of PTB have been relatively under-researched (Keelan, 2011, Rinaldi et al., 2011). The CSAID class of drugs act to specifically target inflammatory signalling pathways, such as NF-κB and P38MAPK, resulting in
inhibition of pro-inflammatory cytokine and prostaglandin secretion without the adverse and non-specific effects of NSAIDs on the fetal ductus arteriosus and renal function (Kaplan et al., 1994, Kaplan et al., 2013, Keelan, 2011, Nakhai-Pour et al., 2011, Sullivan et al., 2002).

We have shown previously that CSAIDs can inhibit production of pro-inflammatory mediators by LPS stimulated choriodecidual cells (De Silva et al., 2010) and explants of human and ovine gestational membranes stimulated ex vivo or in vivo with LPS (Stinson et al., 2014, Sullivan et al., 2002). This study is the first to assess the safety and efficacy of CSAIDs administered IA in a well-established sheep model of preterm chorioamnionitis and FIRS (Kallapur et al., 2007, Kallapur et al., 2001, Polglase et al., 2010, Saito et al., 2014) confirming data from earlier studies in simpler models.

TPCA-1 was administered IA (2.8 mg) to yield an approximate concentration in amniotic fluid of 20 μM. This single IA dose of TPCA-1 was sufficient to inhibit LPS induced amniotic production of PGE₂ and IL-6, but not IL-8, over a 48 h period. Increased amniotic PGE₂ and IL-6 are both associated with IUI and onset of PTL, with increased amniotic IL-6 concentrations being a well-accepted biomarker of PTB and adverse neonatal outcomes (Chaemsaiithong et al., 2014, Menon et al., 2008, Romero et al., 2014). This supports previous studies where TPCA-1 dose-dependently (0.28-35.8 μM) inhibited production of pro-inflammatory mediators by LPS-stimulated primary human choriodecidual cells in culture (IC₅₀ of 7.05 μM for inhibition of IL-6 production and 2.35 μM for inhibition of TNF-α) (De Silva et al., 2010). It also concurs with our previous ex vivo studies which found TPCA-1 (7 μM) inhibited TNF-α and PGE₂ production by E.coli (killed)-stimulated human, term, full-thickness gestational membranes plus PGE₂ and IL-8 production by full-thickness sheep gestational membranes collected from IA LPS and U. parvum stimulated ewes (Stinson
et al., 2014). This is the first study, of which we are aware, to demonstrate the anti-inflammatory effects of TPCA-1 in a live animal model of IUI.

OxZnl is a less well studied fungal inhibitor with specificity for mitogen-activated protein kinase kinase kinase (7MAP3K7), also known as TGF-β-activated kinase 1 (TAK1) (Wu et al., 2013). This enzyme lies upstream of IKKβ in the NF-κB pathway and is also involved in the p38MAPK signalling pathway; hence, it makes an ideal anti-inflammatory drug target. OxZnl, administered IA at a dose of 0.875 mg to yield an approximate concentration in AF of 5 μM was found to be similarly effective as TPCA-1 at reducing concentrations of amniotic PGE2, less effective at inhibiting IL-6 production, and (like TPCA-1) ineffective at inhibiting IL-8 production in response to IA LPS stimulation. This is the first published study, of which we are aware, to assess OxZnl in pregnancy tissues or IUI. In unpublished pilot studies we have observed a dose dependent effect (0.1-10 μM) of OxZnl on IL-6 production by E.coli-stimulated primary human decidual cells in culture (IC50 of ~1 μM). In support of the AF PGE2 and IL-6 data, we found a trend towards a lower membrane inflammatory score (measure of histologic chorioamnionitis) in TPCA-1 and OxZnl treated animals compared.

As a secondary outcome we assessed fetal effects of IA CSAID delivery and evidence of fetal toxicity. Both TPCA-1 and OxZnl appeared to be reasonably well tolerated by the preterm sheep fetus, at least up to 48 h exposure. There were only minor differences in fetal cord blood gases, fetal cord blood cell counts or liver function tests between the groups. There was minimal evidence for O2/CO2 compensation in the LPS/OxZnl group as evidenced by increased pO2 and no significant changes in pH or albumin levels, although the small sample size limits the strength of these observations. Further toxicity studies are required over a longer duration and in the absence of LPS, although adverse effects are more likely to be
apparent in the presence of LPS. Whilst the 2d LPS model of chorioamnionitis is limited in
that vigorous fetal inflammatory responses take longer to develop, we did find modest
increases in fetal circulating cytokine/chemokine levels, fetal lung cytokine/chemokine
mRNA expression, inflammatory infiltration and increased liver acute-phase protein mRNA
expression evident at the 48 h time-point. Fetal lung PMN cell infiltration was the only
inflammatory response which appeared to be inhibited at this time point by a CSAID,
specifically by OxZnl. This may reflect the fact that the lungs are exposed to the CSAIDs
directly via the AF, whereas systemic exposure requires absorption via the gut which is
unlikely to result in therapeutic levels being achieved following a single low dose. Longer
time course studies are now required to fully determine the safety and efficacy of these
CSAIDs on established fetal inflammatory responses in either this LPS-chorioamnionitis
model or a true infection model. As fetal sheep swallow and breathe in amniotic fluid in the
order of ~100-500 ml per day (Tomoda et al., 1985, Underwood et al., 2005) future
pharmaco/toxicokinetic studies must consider the activity, half-life and metabolism of these
CSAIDs over time, fetal uptake, localisation of the inhibitory effect, and potential side effects
of long-term in utero CSAID exposure as modulation of fetal inflammatory signalling may
effect normal fetal development (Arsenescu et al., 2011, Hayden et al., 2006).

Finally, maternal exposure to immune-modulating therapies may increase risk of maternal
infection. We have previously shown that the anti-inflammatory effects of TPCA-1 in a
human gestational membrane Transwell model are primarily restricted to the fetal face of the
membranes (amniotic compartment) (Stinson et al., 2014) suggesting minimal risk of
maternal exposure. This possibility is even more unlikely after taking into account the dose
administered IA and its achievable concentrations in maternal circulation. In this study, we
found minimal effects of TPCA-1 and OxZnl on maternal circulating cytokine/chemokine
levels suggesting that maternal plasma concentrations remained low and sub-therapeutic. However, we did find evidence of lower circulating maternal IL-6 concentrations in the CSAID-treated groups. Additional studies are needed to confirm this and ascertain the mechanism by which this effect may be mediated.

The primary limitation of this study was the small samples size. With a starting value of N=7 and a fetal loss rate of 18% we had only 4 animals left in the LPS/OxZnl group. Furthermore, the invasive nature and complexity of the chronically-catheterised model meant collection of AF and maternal and fetal blood at every time point from every animal was not always possible. Unavailability of missing data points meant that repeated measures statistical analyses could not be performed in the time-course study. As discussed above, the limitation of the 2d LPS-chorioamnionitis model meant that the inhibitory effects of the CSAIDs could not be fully tested on established fetal inflammation. It is likely that optimal clinical benefits will be achieved if both amniotic and fetal inflammation are significantly reduced by CSAID treatment. It remains to be seen whether higher concentrations or repeated doses of the CSAIDs result in more dramatic anti-inflammatory effects within the amniotic cavity, fetal lungs and circulation, although risk of side effects may also increase.

In conclusion, prophylactic antibiotics given to women at high risk of IUI have had limited success in preventing PTB or improving neonatal outcomes. This is likely due in part to the choice and timing of antibiotic administered, the selection of patients deemed ‘at risk’, and the pro-inflammatory nature of microbial lysis (Joergensen et al., 2014b, Kenyon et al., 2008a, 2008b, Kenyon et al., 2001a, 2001b, Simcox et al., 2007). Our findings that IA-delivered TPCA-1 and OxZnl inhibit intraamniotic inflammation induced by LPS (a widely-used microbial stimulus) without evidence of overt toxicity support our contention that IA
administration of CSAIDs, in conjunction with an appropriate and effective antibiotic regimen, could offer a significant therapeutic advantage over antibiotics alone to treat and prevent infection/inflammation-driven PTB. Whilst we observed little evidence for significant differences in efficacy between the two CSAIDs tested, it is likely that improved efficacy of both will be required to achieve significant clinical benefits and the prevention of FIRS. Additional exploration of this approach is warranted to further evaluate the benefits and risks of this therapy, improve efficacy in both the amniotic and fetal compartments, and establish a safe and effective dose and administration regimen for pre-clinical evaluation. Whilst future studies must closely assess long-term fetal outcomes post CSAID treatment to ensure no adverse effects of NF-κB blockade on fetal development, the present findings support our primary hypothesis and provide rationale for the further investigation of these and other compounds, in combination with antibiotics, for the prevention of IUI and subsequent PTB.

Authors’ roles
DJI performed the studies, analysed the samples and data, and wrote the manuscript. YM performed the PCR analyses. MS and MWK designed and performed the animal studies. JAK conceived, designed and funded the study in conjunction with JPN and MWK. All authors contributed to the writing and editing of the manuscript.

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Conflict of Interest

The authors report no conflicts of interest.
References


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Figure Legends

Figure 1: Experimental design.
Surgery with catheterisation was performed on day 110 of gestation. Five days later, at 116 days’ gestation, three groups of chronically catheterized pregnant ewes received intraamniotic (IA) LPS. A fourth group of unstimulated animals received saline (vehicle controls). One LPS stimulated group received IA TPCA-1; the second LPS stimulated group received IA OxZnl; and the third LPS stimulated group and the unstimulated group (vehicle control) received IA DMSO. Maternal and fetal arterial blood and AF were collected before administration of the LPS and CSAIDs. Samples were then taken at 2, 6, 12, 24, and 48 h post-administration. Fetuses were surgically delivered 48 h post administration and fetal membranes, lung and liver sampled for histology and PCR. Fetal arterial PO$_2$, PCO$_2$ and pH were measured and fetal organs were sampled.

Figure 2: Accumulation of PGE$_2$, IL-6 and IL-8 in amniotic fluid.
Data are box plots with median and whiskers representing 5$^{th}$ - 95$^{th}$ percentiles over a time course of 48 h from LPS stimulation and CSAID treatment. Data were compared for effects of time and treatment by two-way ANOVA and then at each time point by Kruskal-Wallis test. $^a$ significantly different (p<0.05) vs. vehicle control; $^b$ significantly different (p<0.01) vs. control (vehicle only); and $^c$ significantly different (p<0.05) vs. LPS/vehicle at the same time point.

Figure 3: Inflammatory infiltration of gestational membranes.
Gestational membranes 48 h post-LPS stimulation with/without CSAID treatment were stained by H&E and scored for inflammatory infiltrate. A) Representative images are
included for each treatment group; scale bar = 200 μm. B) Individual scores are presented with median for each group. The effect of LPS stimulation (combined groups) was determined vs. control (vehicle only) by Mann Whitney test. Group effects were determined by Kruskal-Wallis test. \(^a\) individual group significantly different (p<0.05) vs. control; \(^b\) combined groups significantly different (p<0.05) vs. control.

**Figure 4: Accumulation of IL-6 and IL-8 in A) maternal and B) fetal plasma.**

Data are box plots with median and whiskers representing 5th-95th percentiles over a time course of 48 h from LPS stimulation and CSAID treatment. Data were compared for effects of time and treatment by two-way ANOVA and then at each time point by Kruskal-Wallis test. \(^a\) significantly different (p<0.05) vs. control (vehicle only) at the same time point.

**Figure 5: Inflammatory infiltration of fetal lung.**

Right upper lobes of fetal lung at 48 h from LPS stimulation and CSAID treatment were stained by H&E and scored for inflammatory infiltrate. A) Representative images from each group are shown: scale bar = 200 μm; arrows point to foci of PMN in alveolar spaces. B) Individual scores are presented with median for each group. The effect of LPS stimulation (combined groups) was determined vs. control (vehicle only) by unpaired t-test. Groups were compared for effects by Kruskal-Wallis test. \(^a\) individual group significantly different (p<0.01) vs. control; \(^b\) individual group significantly different (p<0.05) vs. control; \(^c\) combined LPS stimulated groups significantly different (p<0.05) vs. control.
Figure 6: mRNA expression of pro-inflammatory cytokines and chemokines in fetal lung.

mRNA expression of pro-inflammatory cytokines and chemokines in fetal lung at 48 h from LPS stimulation and CSAID treatment. The Cq values were normalized to 18s rRNA and expressed as fold changes relative to pooled control values. Individual data points are shown together with median for each group. The effect of LPS stimulation (combined groups) on mRNA expression was determined vs. control (vehicle only) by unpaired t-test for normally distributed data and by Mann Whitney test for non-parametric data. Groups were compared for effects by Kruskal-Wallis test. 

- individual group significantly different (p<0.05) vs. control; 
- combined groups significantly different (p<0.05) vs. control.

Figure 7: mRNA expression of acute-phase proteins in fetal liver.

mRNA expression of acute-phase proteins in fetal liver at 48 h from LPS stimulation and CSAID treatment. The Cq values were normalized to 18s rRNA and expressed as fold changes relative to pooled control values. Individual data points are shown together with median for each group. The effect of LPS stimulation (pooled groups) on mRNA expression was determined vs. control (vehicle only) by unpaired t-test for normally distributed data and by Mann Whitney test for non-parametric data. Groups were compared for effects by Kruskal-Wallis test.

- individual group significantly different (p<0.05) vs. control; 
- combined groups significantly different (p<0.05) vs. control; 
- combined groups marginally significant (p=0.05) vs. control.
Table I: Birth weight and fetal arterial cord blood measurements at delivery.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle control N=6</th>
<th>LPS/vehicle N=6</th>
<th>LPS/OxZnl N=4</th>
<th>LPS/TPCA-1 N=6</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Birth weight (kg)</strong></td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>0.77</td>
</tr>
<tr>
<td><strong>Cord blood gases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>— pH</td>
<td>7.1 ± 0.03</td>
<td>7.1 ± 0.0</td>
<td>7.0 ± 0.04</td>
<td>7.1 ± 0.06</td>
<td>0.55</td>
</tr>
<tr>
<td>— pCO2 (mmHg)</td>
<td>91.3 ± 8.9</td>
<td>88.6 ± 22.3</td>
<td>103.8</td>
<td>89.0 ± 8.6</td>
<td>0.74</td>
</tr>
<tr>
<td>— pO2 (mmHg)</td>
<td>10.5 ± 5.0</td>
<td>11.6 ± 2.8</td>
<td>22.8 ± 1.5 a</td>
<td>12.4 ± 3.7</td>
<td>0.04</td>
</tr>
<tr>
<td>— HCO3⁻ act (mmol/L)</td>
<td>24.9 ± 1.9</td>
<td>24.5 ± 4.2</td>
<td>24.0</td>
<td>26.2 ± 3.0</td>
<td>0.86</td>
</tr>
<tr>
<td>— Base excess (B) mmol/L</td>
<td>-6.8 ± 2.3</td>
<td>-7.35 ± 3.3</td>
<td>-8.7</td>
<td>-4.8 ± 3.8</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>Cord blood white cell counts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>— Haemoglobin (g/L)</td>
<td>119 ± 11</td>
<td>109 ± 4</td>
<td>111 ± 9</td>
<td>110 ± 10</td>
<td>0.30</td>
</tr>
<tr>
<td>— Total WBC (10⁹/L)</td>
<td>4.0 ± 0.8</td>
<td>2.4 ± 1.3</td>
<td>3.2 ± 1.8</td>
<td>3.0 ± 2.2</td>
<td>0.39</td>
</tr>
<tr>
<td>— Neutrophils (10⁹/L)</td>
<td>0.9 ± 0.3</td>
<td>0.3 ± 0.1 b</td>
<td>0.5 ± 0.6</td>
<td>0.2 ± 0.2 b</td>
<td>0.003</td>
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<td>— Lymphocytes (10⁹/L)</td>
<td>2.3 ± 0.8</td>
<td>1.3 ± 0.7</td>
<td>1.6 ± 1.0</td>
<td>2.0 ± 1.3</td>
<td>0.46</td>
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<td>— Monocytes (10⁹/L)</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.05</td>
<td>0.3 ± 0.3</td>
<td>0.1 ± 0.2</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Data are mean ± SD. Fetal cord gas data available for only N=4 vehicle control, N=2 LPS/vehicle, N=1-2 LPS/OxZnl and N=3-4 LPS/TPCA-1. a p<0.05 vs. control (vehicle only); b p<0.01 vs. control by one-way ANOVA and post-hoc Tukey’s multiple comparison test. Abbreviations: N, group size; WBC, white blood cell.
Table II: Fetal liver function tests at delivery.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle control N=6</th>
<th>LPS/vehicle N=6</th>
<th>LPS/OxZnl N=4</th>
<th>LPS/TPCA-1 N=6</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>22 ± 10</td>
<td>24 ± 10</td>
<td>18 ± 0</td>
<td>20 ± 4</td>
<td>0.71</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>22 ± 11</td>
<td>22 ± 2</td>
<td>26 ± 12</td>
<td>30 ± 18</td>
<td>0.71</td>
</tr>
<tr>
<td>GLDH (U/L)</td>
<td>4 ± 5</td>
<td>3 ± 2</td>
<td>4 ± 5</td>
<td>4 ± 4</td>
<td>0.95</td>
</tr>
<tr>
<td>Total bilirubin (μmol/L)</td>
<td>13 ± 5</td>
<td>12 ± 2</td>
<td>11 ± 3</td>
<td>13 ± 4</td>
<td>0.82</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>21 ± 1</td>
<td>19 ± 1</td>
<td>19 ± 1 a</td>
<td>20 ± 1</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Data are mean ± SD. *p<0.05 vs. control (vehicle only) by one-way ANOVA and post-hoc Tukey’s multiple comparison test. Abbreviations: N, group size; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; GLDH, glutamate dehydrogenase.