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

1 **Introduction**

2

3 Preterm birth (PTB), defined as delivery less than 37 weeks of gestation, accounts for
4 approximately 11% of all live births worldwide (Blencowe *et al.*, 2013) and is associated
5 with 70% of all neonatal mortality and morbidity (Goldenberg *et al.*, 2008). The morbidity
6 and mortality associated with PTB incurs significant human and financial costs (Petrou,
7 2005); the annual societal costs associated with PTB in the USA was estimated to be $26.2
8 billion in 2005 (Institute of Medicine (US) Committee on Understanding Premature Birth and
9 Assuring Healthy Outcomes, 2007). Despite years of research into the mechanisms
10 responsible for activation of preterm labour (PTL), and the introduction of medical
11 interventions to prevent PTB, rates have continued to increase in developed countries
12 (Blencowe *et al.*, 2013).

13

14 Intrauterine inflammation (IUI) associated with infection is the major cause of early PTB
15 (less than 32 weeks' gestation) and accounts for approximately 40% of all spontaneous PTBs
16 (DiGiulio *et al.*, 2010, Kim *et al.*, 2009, Romero *et al.*, 2001). Activation of pathogen
17 recognition receptors by bacteria, most commonly originating and ascending from the vagina
18 (DiGiulio, 2012, DiGiulio *et al.*, 2010, Jones *et al.*, 2009), initiates a cascade of pro-
19 inflammatory events, resulting in histologic chorioamnionitis and elevated amniotic fluid
20 (AF) concentrations of pro-inflammatory cytokines, chemokines and prostaglandins
21 (Agrawal *et al.*, 2012, Elovitz *et al.*, 2003, Romero *et al.*, 2001). These events trigger uterine
22 contractions and initiation of PTL. In the most severe cases, IUI can lead to fetal
23 inflammatory response syndrome (FIRS) (reviewed in (Gotsch *et al.*, 2007)) characterised by
24 increased fetal expression of chemokines and cytokines and damage to the cardiovascular,

1 pulmonary, hepatic, gastrointestinal and neurological systems (Fawke, 2007, Jobe, 2012,
2 Lahra *et al.*, 2009, Shatrov *et al.*, 2010, Viscardi *et al.*, 2004).
3
4 The administration of antibiotics to women for the treatment or prevention of preterm labour
5 has been shown to have little effect on reducing rates of preterm birth or neonatal morbidity
6 and mortality (Joergensen *et al.*, 2014a, Kenyon *et al.*, 2008a, 2008b, Kenyon *et al.*, 2001a,
7 2001b, Simcox *et al.*, 2007). As maternal and fetal inflammation associated with IUI is
8 usually well established at presentation with PTL, effective treatment is required to both
9 combat the infection and reduce the associated inflammatory response (Keelan, 2011, Rinaldi
10 *et al.*, 2011). In an important proof-of-principle study, co-treatment of a high risk cohort of
11 women with bacterial vaginosis with N-acetyl cysteine (NAC), a non-specific inhibitor of
12 inflammatory signalling pathways, was shown to reduce the incidence of PTB, increase birth
13 weight and reduce neonatal morbidity/mortality over and above the effects of 17-
14 hydroxyprogesterone caproate alone (Shahin *et al.*, 2009). However, NAC therapy has not
15 progressed into mainstream clinical use, likely in part because it is poorly tolerated (Shahin *et*
16 *al.*, 2009) and its efficacy has yet to be confirmed in other studies.
17
18 Non-steroidal anti-inflammatory drugs (NSAIDS) were once commonly prescribed in
19 pregnancy to treat fever, pain and inflammation, but their use has declined due to adverse
20 effect profiles (Kaplan *et al.*, 1994, Kaplan *et al.*, 2013, Nakhai-Pour *et al.*, 2011). Cytokine-
21 suppressive anti-inflammatory drugs (CSAIDs) are a novel category of anti-inflammatory
22 drugs that specifically target inflammatory signalling pathways (e.g. NF-κB or MAPK
23 pethways), without the adverse and/or non-specific effects of NSAIDs (Griswold *et al.*, 1993,
24 Keelan, 2011). We recently compared the anti-inflammatory effects of NAC and three
25 CSAIDs on stimulated human and ovine gestational membranes and found two drugs were

1 effective at suppressing cytokine and prostaglandin production *in vitro* (Stinson *et al.*, 2014):
2 an IKKβ inhibitor TPCA-1 (5-[p-fluorophenyl)-2-ureido] thiophene-3-carboxamide) (De
3 Silva *et al.*, 2010) and a MAPK inhibitor SB239063 (trans-1-[4-hydroxycyclohexyl]-4-[4-
4 fluorophenyl]-5-[2-[methoxy]pyrim-idin-4-yl] imidazole) (Barone *et al.*, 2001, Lappas *et al.*,
5 2007). These data corroborated a much earlier study which demonstrated SKF86002, a
6 CSAID prototype, inhibited levels of IL-1β protein, COX-2 mRNA expression and PGE_2
7 production by full-thickness human gestational membranes stimulated *in vitro* with LPS
8 (Sullivan *et al.*, 2002). While the *in vitro* evidence suggests further investigation of CSAIDs
9 for the prevention of inflammation-mediated PTB is warranted, it is unknown to what extent
10 NF-κB/MAPK inhibitors would be safe and effective *in vivo* in terms of preventing preterm
11 labour and/or treating FIRS.
12
13 The size and the gestational length (approximately 147 days) of sheep enables longitudinal
14 pregnancy studies including non-invasive amniotic fluid sampling under ultrasound guidance,
15 and surgical interventions such as long-term catheterization (reviewed in (Kemp *et al.*, 2010).
16 Intraamniotic LPS administration in sheep results in chorioamnionitis, elevated
17 proinflammatory cytokine expression in the amnion/chorion, increased cytokine and
18 prostaglandin levels in the amniotic fluid and fetal lung inflammation (Kallapur *et al.*, 2001)
19 mimicking that observed in the human condition and making it a good experimental model
20 for targetting inflammatory responses in pregnancy. Although intraamniotic injection with
21 LPS or live microorganisms does not induce preterm labour in sheep (Moss *et al.*, 2005), this
22 sheep model of IUI is ideal for pharmacological intervention studies, as it enables sustained
23 longitudinal assessments without the complication imposed by premature labour and
24 delivery. The present study, therefore, aimed to assess in an ovine model of LPS-induced
25 chorioamnionitis the IA anti-inflammatory efficacies of two CSAIDs: TPCA-1 and 5z-7-

1 oxozeaenol (OxZnl), a selective fungal inhibitor of TAK1 (transforming growth factor beta-
2 activated kinase 1) kinase and ATPase activity (Wu et al., 2013). We employed intraamniotic
3 (IA) CSAID delivery to allow localised targeting of the gestational membranes and the fetus
4 (the key sites of IA infection-driven inflammation) and minimise the risk of unintended
5 maternal immune modulation. We hypothesised that IA-administered CSAIDs would be
6 inhibit LPS-induced inflammation within the amniotic cavity and gestational membranes,
7 with lesser or negligible effects on the fetus and mother.

8

9 **Methods**

10

11 **Animals, surgical procedures and CSAID administration**

12 All experimental procedures were approved by the Animal Ethics Committee of The
13 University of Western Australia (RA/3/100/1203). Animal management, anaesthesia, surgical
14 catheterisation, and recovery have all been previously described (Kemp *et al.*, 2013). The
15 experimental model is summarised diagrammatically in Figure 1). Surgery with
16 catheterisation was performed on day 110 of gestation. At 116 days of gestation (equivalent
17 to approx. 32 weeks of human gestation), three groups of chronically catheterized pregnant
18 ewes received an intraamniotic (IA) bolus of 10 mg LPS from *E.coli* O55:B5 (LPS; L2880,
19 Sigma Aldrich, St. Louis, MO) in 2 ml saline. Inflammatory effects of 48 h IA 10 mg LPS
20 stimulation have been previously described (Kallapur *et al.*, 2007, Saito *et al.*, 2014). A
21 fourth group of unstimulated animals received IA 2 ml saline (vehicle controls). One LPS
22 stimulated group received IA 2.8 mg TPCA-1 (Merck Millipore, Darmstadt, Germany) in 1
23 ml dimethyl sulfoxide vehicle (DMSO; D8418, Sigma Aldrich); the second LPS stimulated
24 group received IA 0.875 mg OxZnl (BioAustralis Fine Chemicals, Smithfield, Australia) in 1
25 ml DMSO, and the third LPS stimulated group received IA 1 ml DMSO. The unstimulated

1 group (vehicle control) also received IA 1 ml DMSO. TPCA-1 was administered IA to yield
2 an approximate concentration of 20 μM in AF based on an approximate volume of 500 ml at
3 115 days sheep gestation (equivalent to 30 weeks' human gestation) (Sandlin *et al.*, 2014,
4 Tomoda *et al.*, 1985). OxZnl was administered to yield an approximate concentration of 5
5 μM in AF. The target doses were chosen based on concentration-response data from our
6 preliminary and published fetal membrane studies; these studies indicated that concentrations
7 of 20 μM TPCA-1 and 5 μM OxZnl are close to maximally effective in suppressing cytokine
8 production without exerting toxicity (De Silva *et al.*, 2010, Stinson *et al.*, 2014). Maternal
9 and fetal arterial blood and AF (two sets of 1 ml samples) were collected into heparinized
10 tubes 30 min before administration of the LPS and CSAIDs. Samples were then also taken at
11 2, 6, 12, 24, and 48 h post-administration, by which time the effects of the CSAIDs were
12 expected to have plateaued. Fetal arterial pH, PO_2, PCO_2, HCO_3^- and base excess (BE) were
13 measured with a Rapid Lab 1265 blood gas analyser (Siemens, Germany). Plasma and AF
14 samples were stored at −80°C for analysis.

15

16 **Haematology and liver function tests**

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

23

24

25

1 **Measurement of cytokine and PGE_2 concentrations**

2 Ovine IL-6 and IL-8 protein concentrations in AF and maternal and fetal plasma were
3 determined by ELISA as previously described (Saito *et al.*, 2014). Samples were analysed
4 undiluted for IL-6 and at 1:50 dilution for IL-8. The top standard for IL-6 was 8000 pg/ml
5 with a lower limit of detection of 150 pg/ml and average intra-assay coefficient of variability
6 (CV) of 8%. The top standard for IL-8 was 8000 pg/ml with a lower limit of detection of 25
7 pg/ml and CV of 10%. Quantification of PGE_2 in AF was performed using a prostaglandin
8 E_2 EIA Kit – monoclonal (Cayman Chemical Company, Ann Arbor, MI, USA) with
9 unstimulated samples analysed at 1:10 dilution, LPS-stimulated samples at 1:250 dilution and
10 LPS/CSAID samples at 1:20 dilution. The assay range PGE_2 was 10-1000 pg/ml with a CV
11 of 9%. Samples falling below the lower limits of detection were assigned the concentration
12 equivalent to the lower limit detected for statistical analysis.

13

14 **Relative Quantification of mRNA Expression**

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

1 SD for the four groups = 14.82 ± 0.10, 14.81 ± 0.03, 14.81 ± 0.08, 14.92 ± 0.05,
2 respectively).
3
4 **Fetal lung and membrane histology**
5 Sections (5 µm thick) from formalin-fixed paraffin-embedded amnio-chorion and fetal lung
6 (right upper lobe) were stained with haematoxylin and eosin (H&E). Stained sections were
7 imaged using an Aperio ScanScope XT (Leica Biosystems, Nussloch, Germany) with 40×
8 objective and imaged at 10× magnification using ImageScope version 11.2.0.780 (Aperio
9 Technologies Inc. Leica Biosystems, Nussloch, Germany). Five random fields were assessed
10 at low-power magnification and scored as previously described (Kemp *et al.*, 2014). The
11 scorer was blind to the group identity of the sections.
12
13 For fetal lung, scores were as follows:
14 0, normal
15 1, few airspace inflammatory cells and no consolidation
16 2, foci of airspace inflammatory cells with minimal microconsolidation
17 3, foci of airspace inflammatory cells with microconsolidation
18 4: extensive airspace inflammatory cells and consolidation
19 For chorion:
20 0, no chorioamnionitis
21 1, few polymorphonuclear (PMN) cells in subchorionic fibrin and/or membrane
22 trophoblast layer (stage 1, grade 1 chorioamnionitis)
23 2, isolated foci or bands of PMN cells in subchorionic fibrin and/or membrane
24 trophoblast layer (stage 1, grade 2 chorioamnionitis)

1 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 PGE_2 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

1 (birth weight at delivery 1.3 kg). The fetal loss rate for catheterised animals was 11% (3/28
2 animals). Two fetuses died following administration of LPS and pharmacological agents (one
3 LPS/TPCA-1 and one LPS/OxZnl). In both cases the fetuses were anatomically normal, with
4 no signs of infection and all surgical installations intact; these animals were excluded from
5 analyses. One fetus (LPS/OxZnl) was found dead mid-sampling, likely from exsanguination
6 as a result of a torn fetal carotid catheter, and was excluded from analyses. None of these
7 deaths appeared to be specifically attributed to the CSAID treatments. A final sample size of
8 N=6 vehicle control, N=6 LPS/vehicle, N=4 LPS/OxZnl and N=6 LPS/TPCA-1 was
9 available for study.
10
11 **Effect of CSAIDS on intraamniotic inflammation**
12 Intraamniotic administration of LPS (LPS/vehicle) stimulated PGE_2 production in AF
13 (treatment factor p=0.0002 by two-way ANOVA; **Figure 2**). PGE_2 levels in AF were
14 significantly increased at each time point in LPS/vehicle animals compared to unstimulated
15 vehicle controls ($p<0.05$ by Kruskal-Wallis test). Co-administration of TPCA-1 or OxZnl
16 abrogated the stimulatory effects of LPS on production of PGE_2, resulting in the loss of
17 significance compared to vehicle control. Intraamniotic IL-6 concentrations were increased
18 with LPS stimulation (treatment factor p=0.029). Whilst there was a trend towards reduced
19 IL-6 levels with CSAID treatments, particularly prior to 12 h, this was not statistically
20 significant at individual time points (**Figure 2**). Intraamniotic IL-8 production increased
21 marginally over time in response to LPS stimulation (time factor p=0.002 and treatment
22 factor p=0.072), with a statistically significant increase in IL-8 concentrations observed at 24
23 h with LPS stimulation ($p<0.05$ *vs*. vehicle control). Although median IL-8 levels appeared
24 lower in the CSAID groups, particularly at early time points, the variability was considerable
25 and differences were not statistically significant.

1

2 Analysis of H&E stained gestational membranes from LPS-stimulated animals demonstrated
3 significant infiltration of PMN cells compared to vehicle controls ($p=0.0007$ by t-test for
4 combined LPS animals *vs*. vehicle control; **Figure 3**). PMN infiltration score in the
5 LPS/vehicle group was significantly increased *vs*. vehicle control ($p<0.01$). This difference
6 was abolished with TPCA-1 and OxZnl treatments.

7

8 **Effects of CSAIDs on the preterm fetus**

9 Birth weight (g) was similar across the four groups (**Table I**). Fetal arterial cord total white
10 blood cell numbers, lymphocyte numbers and monocyte numbers were comparable across
11 groups at 48 h. The number of neutrophils was reduced in fetal arterial cord blood of all LPS-
12 stimulated groups, although this difference only reached significance for the LPS/vehicle and
13 LPS/TPCA-1 groups ($p<0.01$ *vs*. vehicle control). There were no significant differences in
14 fetal arterial cord blood pH, pCO_2, HCO_3^-, or BE(B) between groups. pO_2 was increased in
15 the LPS/OxZnl group ($p<0.05$) *vs*. vehicle control. Fetal liver function tests revealed a
16 marginal reduction in albumin concentration ($p<0.05$) in LPS/OxZnl animals compared to
17 vehicle controls (**Table II**). Concentrations of liver enzymes (AST, aspartate
18 aminotransferase; GGT, gamma glutamyl transpeptidase; GLDH, glutamate dehydrogenase)
19 and total bilirubin were not different between groups.

20

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

22 Maternal plasma concentrations of IL-8 were increased in LPS-stimulated animals (treatment
23 factor $p=0.027$), particularly in the first 12 h. No significant inhibitory effect of either CSAID
24 treatment was observed (**Figure 4A**). A significant increase in maternal circulating IL-6 was
25 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.

1 Modest increases in acute-phase protein mRNA expression were detected at delivery in fetal
2 liver of LPS stimulated animals *vs*. vehicle controls (**Figure 7**). Increases of ~2-fold for CRP
3 (p=0.057, t-test) and Hepcidin (p=0.027, t-test) were observed, while a much more robust
4 increase of ~180-fold was detected for SAA (p=0.0003, Mann Whitney). Only SAA
5 expression was significantly increased in individual LPS-stimulated groups (LPS/TPCA-1
6 and LPS/OxZnl) *vs.* vehicle control ($p<0.05$). There was no evidence for inhibition of mRNA
7 expression by CSAID treatment at the 48 h time point.

8

9 **Discussion**

10

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

21

22 Despite the knowledge of the importance of inflammation as a cause of PTB, anti-
23 inflammatory modalities for the treatment or prevention of PTB have been relatively under-
24 researched (Keelan, 2011, Rinaldi *et al.*, 2011). The CSAID class of drugs act to specifically
25 target inflammatory signalling pathways, such as NF-κB and P38MAPK, resulting in

1 inhibition of pro-inflammatory cytokine and prostaglandin secretion without the adverse and
2 non-specific effects of NSAIDs on the fetal ductus arteriosus and renal function (Kaplan *et*
3 *al.*, 1994, Kaplan *et al.*, 2013, Keelan, 2011, Nakhai-Pour *et al.*, 2011, Sullivan *et al.*, 2002).
4 We have shown previously that CSAIDs can inhibit production of pro-inflammatory
5 mediators by LPS stimulated choriodecidual cells (De Silva *et al.*, 2010) and explants of
6 human and ovine gestational membranes stimulated *ex vivo* or *in vivo* with LPS (Stinson *et*
7 *al.*, 2014, Sullivan *et al.*, 2002). This study is the first to assess the safety and efficacy of
8 CSAIDs administered IA in a well-established sheep model of preterm chorioamnionitis and
9 FIRS (Kallapur *et al.*, 2007, Kallapur *et al.*, 2001, Polglase *et al.*, 2010, Saito *et al.*, 2014)
10 confirming data from earlier studies in simpler models.
11
12 TPCA-1 was administered IA (2.8 mg) to yield an approximate concentration in amniotic
13 fluid of 20 μM. This single IA dose of TPCA-1 was sufficient to inhibit LPS induced
14 amniotic production of PGE_2 and IL-6, but not IL-8, over a 48 h period. Increased amniotic
15 PGE_2 and IL-6 are both associated with IUI and onset of PTL, with increased amniotic IL-6
16 concentrations being a well-accepted biomarker of PTB and adverse neonatal outcomes
17 (Chaemsaithong *et al.*, 2014, Menon *et al.*, 2008, Romero *et al.*, 2014). This supports
18 previous studies where TPCA-1 dose-dependently (0.28-35.8 μM) inhibited production of
19 pro-inflammatory mediators by LPS-stimulated primary human choriodecidual cells in
20 culture (IC_{50} of 7.05 μM for inhibition of IL-6 production and 2.35 μM for inhibition of
21 TNF-α) (De Silva *et al.*, 2010). It also concurs with our previous *ex vivo* studies which found
22 TPCA-1 (7 μM) inhibited TNF-α and PGE_2 production by *E.coli* (killed)-stimulated human,
23 term, full-thickness gestational membranes plus PGE_2 and IL-8 production by full-thickness
24 sheep gestational membranes collected from IA LPS and *U. parvum* stimulated ewes (Stinson

1 *et al.*, 2014). This is the first study, of which we are aware, to demonstrate the anti-
2 inflammatory effects of TPCA-1 in a live animal model of IUI.
3
4 OxZnl is a less well studied fungal inhibitor with specificity for mitogen-activated protein
5 kinase kinase kinase (7MAP3K7), also known as TGF-β-activated kinase 1 (TAK1) (Wu *et*
6 *al.*, 2013). This enzyme lies upstream of IKKβ in the NF-κB pathway and is also involved in
7 the p38MAPK signalling pathway; hence, it makes an ideal anti-inflammatory drug target.
8 OxZnl, administered IA at a dose of 0.875 mg to yield an approximate concentration in AF of
9 5 μM was found to be similarly effective as TPCA-1 at reducing concentrations of amniotic
10 PGE_2, less effective at inhibiting IL-6 production, and (like TPCA-1) ineffective at inhibiting
11 IL-8 production in response to IA LPS stimulation. This is the first published study, of which
12 we are aware, to assess OxZnl in pregnancy tissues or IUI. In unpublished pilot studies we
13 have observed a dose dependent effect (0.1-10 μM) of OxZnl on IL-6 production by *E.coli*-
14 stimulated primary human decidual cells in culture (IC_{50} of ~1 μM). In support of the AF
15 PGE_2 and IL-6 data, we found a trend towards a lower membrane inflammatory score
16 (measure of histologic chorioamnionitis) in TPCA-1 and OxZnl treated animals compared.
17
18 As a secondary outcome we assessed fetal effects of IA CSAID delivery and evidence of fetal
19 toxicity. Both TPCA-1 and OxZnl appeared to be reasonably well tolerated by the preterm
20 sheep fetus, at least up to 48 h exposure. There were only minor differences in fetal cord
21 blood gases, fetal cord blood cell counts or liver function tests between the groups. There was
22 minimal evidence for O_2/CO_2 compensation in the LPS/OxZnl group as evidenced by
23 increased pO_2 and no significant changes in pH or albumin levels, although the small sample
24 size limits the strength of these observations. Further toxicity studies are required over a
25 longer duration and in the absence of LPS, although adverse effects are more likely to be

1 apparent in the presence of LPS. Whilst the 2d LPS model of chorioamnionitis is limited in
2 that vigorous fetal inflammatory responses take longer to develop, we did find modest
3 increases in fetal circulating cytokine/chemokine levels, fetal lung cytokine/chemokine
4 mRNA expression, inflammatory infiltration and increased liver acute-phase protein mRNA
5 expression evident at the 48 h time-point. Fetal lung PMN cell infiltration was the only
6 inflammatory response which appeared to be inhibited at this time point by a CSAID,
7 specifically by OxZnl. This may reflect the fact that the lungs are exposed to the CSAIDs
8 directly via the AF, whereas systemic exposure requires absorption via the gut which is
9 unlikely to result in therapeutic levels being achieved following a single low dose. Longer
10 time course studies are now required to fully determine the safety and efficacy of these
11 CSAIDs on established fetal inflammatory responses in either this LPS-chorioamnionitis
12 model or a true infection model. As fetal sheep swallow and breathe in amniotic fluid in the
13 order of ~100-500 ml per day (Tomoda *et al.*, 1985, Underwood *et al.*, 2005) future
14 pharmaco/toxicokinetic studies must consider the activity, half-life and metabolism of these
15 CSAIDs over time, fetal uptake, localisation of the inhibitory effect, and potential side effects
16 of long-term *in utero* CSAID exposure as modulation of fetal inflammatory signalling may
17 effect normal fetal development (Arsenescu *et al.*, 2011, Hayden *et al.*, 2006).
18
19 Finally, maternal exposure to immune-modulating therapies may increase risk of maternal
20 infection. We have previously shown that the anti-inflammatory effects of TPCA-1 in a
21 human gestational membrane Transwell model are primarily restricted to the fetal face of the
22 membranes (amniotic compartment) (Stinson *et al.*, 2014) suggesting minimal risk of
23 maternal exposure. This possibility is even more unlikely after taking into account the dose
24 administered IA and its achievable concentrations in maternal circulation. In this study, we
25 found minimal effects of TPCA-1 and OxZnl on maternal circulating cytokine/chemokine

1 levels suggesting that maternal plasma concentrations remained low and sub-therapeutic.
2 However, we did find evidence of lower circulating maternal IL-6 concentrations in the
3 CSAID-treated groups. Additional studies are needed to confirm this and ascertain the
4 mechanism by which this effect may be mediated.
5
6 The primary limitation of this study was the small samples size. With a starting value of N=7
7 and a fetal loss rate of 18% we had only 4 animals left in the LPS/OxZnl group. Furthermore,
8 the invasive nature and complexity of the chronically-catheterised model meant collection of
9 AF and maternal and fetal blood at every time point from every animal was not always
10 possible. Unavailability of missing data points meant that repeated measures statistical
11 analyses could not be performed in the time-course study. As discussed above, the limitation
12 of the 2d LPS-chorioamnionitis model meant that the inhibitory effects of the CSAIDs could
13 not be fully tested on established fetal inflammation. It is likely that optimal clinical benefits
14 will be achieved if both amniotic and fetal inflammation are significantly reduced by CSAID
15 treatment. It remains to be seen whether higher concentrations or repeated doses of the
16 CSAIDs result in more dramatic anti-inflammatory effects within the amniotic cavity, fetal
17 lungs and circulation, although risk of side effects may also increase.
18
19 In conclusion, prophylactic antibiotics given to women at high risk of IUI have had limited
20 success in preventing PTB or improving neonatal outcomes. This is likely due in part to the
21 choice and timing of antibiotic administered, the selection of patients deemed 'at risk', and
22 the pro-inflammatory nature of microbial lysis (Joergensen *et al.*, 2014b, Kenyon *et al.*,
23 2008a, 2008b, Kenyon *et al.*, 2001a, 2001b, Simcox *et al.*, 2007). Our findings that IA-
24 delivered TPCA-1 and OxZnl inhibit intraamniotic inflammation induced by LPS (a widely-
25 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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12

13 **Conflict of Interest**

14 The authors report no conflicts of interest.

15

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4

5

6

7

1 **Figure Legends**

2

3 **Figure 1: Experimental design.**

4 Surgery with catheterisation was performed on day 110 of gestation. Five days later, at 116
5 days' gestation, three groups of chronically catheterized pregnant ewes received
6 intraamniotic (IA) LPS. A fourth group of unstimulated animals received saline (vehicle
7 controls). One LPS stimulated group received IA TPCA-1; the second LPS stimulated group
8 received IA OxZnl; and the third LPS stimulated group and the unstimulated group (vehicle
9 control) received IA DMSO. Maternal and fetal arterial blood and AF were collected before
10 administration of the LPS and CSAIDs. Samples were then taken at 2, 6, 12, 24, and 48 h
11 post-administration. Fetuses were surgically delivered 48 h post administration and fetal
12 membranes, lung and liver sampled for histology and PCR. Fetal arterial PO_2, PCO_2 and pH
13 were measured and fetal organs were sampled.

14

15 **Figure 2: Accumulation of PGE_2, IL-6 and IL-8 in amniotic fluid.**

16 Data are box plots with median and whiskers representing 5th - 95th percentiles over a time
17 course of 48 h from LPS stimulation and CSAID treatment. Data were compared for effects
18 of time and treatment by two-way ANOVA and then at each time point by Kruskal-Wallis
19 test. [a] significantly different ($p<0.05$) *vs.* vehicle control; [b] significantly different ($p<0.01$) *vs.*
20 control (vehicle only); and [c] significantly different ($p<0.05$) *vs.* LPS/vehicle at the same time
21 point.

22

23 **Figure 3: Inflammatory infiltration of gestational membranes.**

24 Gestational membranes 48 h post-LPS stimulation with/without CSAID treatment were
25 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.

1 **Figure 6: mRNA expression of pro-inflammatory cytokines and chemokines in fetal**
2 **lung**
3 mRNA expression of pro-inflammatory cytokines and chemokines in fetal lung at 48 h from
4 LPS stimulation and CSAID treatment**.** The Cq values were normalized to 18s rRNA and
5 expressed as fold changes relative to pooled control values. Individual data points are shown
6 together with median for each group. The effect of LPS stimulation (combined groups) on
7 mRNA expression was determined *vs.* control (vehicle only) by unpaired t-test for normally
8 distributed data and by Mann Whitney test for non-parametric data. Groups were compared
9 for effects by Kruskal-Wallis test. [a] individual group significantly different ($p<0.05$) *vs.*
10 control; [b] combined groups significantly different ($p<0.05$) *vs.* control.
11
12 **Figure 7: mRNA expression of acute-phase proteins in fetal liver.**
13 mRNA expression of acute-phase proteins in fetal liver at 48 h from LPS stimulation and
14 CSAID treatment**.** The Cq values were normalized to 18s rRNA and expressed as fold
15 changes relative to pooled control values. Individual data points are shown together with
16 median for each group. The effect of LPS stimulation (pooled groups) on mRNA expression
17 was determined *vs.* control (vehicle only) by unpaired t-test for normally distributed data and
18 by Mann Whitney test for non-parametric data. Groups were compared for effects by
19 Kruskal-Wallis test. [a] individual group significantly different ($p<0.05$) *vs.* control; [b] combined
20 groups significantly different ($p<0.05$) *vs.* control; [c] combined groups marginally significant
21 ($p=0.05$) *vs.* control.
22

1 **Table I: Birth weight and fetal arterial cord blood measurements at delivery.**

	Vehicle control N=6	**LPS/ vehicle N=6**	**LPS/ OxZnl N=4**	**LPS/ TPCA-1 N=6**	**p-value**
Birth weight (kg)	2.3 ± 0.2	2.3 ± 0.3	2.3 ± 0.2	2.2 ± 0.2	0.77
Cord blood gases					
— pH	7.1 ± 0.03	7.1 ± 0.0	7.0 ± 0.04	7.1 ± 0.06	0.55
— pCO2 (mmHg)	91.3 ± 8.9	88.6 ± 22.3	103.8	89.0 ± 8.6	0.74
— pO2 (mmHg)	10.5 ± 5.0	11.6 ± 2.8	22.8 ± 1.5 [a]	12.4 ± 3.7	0.04
— HCO_3^- act (mmol/L)	24.9 ± 1.9	24.5 ± 4.2	24.0	26.2 ± 3.0	0.86
— Base excess (B) mmol/L	-6.8 ± 2.3	-7.35 ± 3.3	-8.7	-4.8 ± 3.8	0.67
Cord blood white cell counts					
— Haemoglobin (g/L)	119 ± 11	109 ± 4	111 ± 9	110 ± 10	0.30
— Total WBC (10^9/L)	4.0 ± 0.8	2.4 ± 1.3	3.2 ± 1.8	3.0 ± 2.2	0.39
— Neutrophils (10^9/L)	0.9 ± 0.3	0.3 ± 0.1 [b]	0.5 ± 0.6	0.2 ± 0.2 [b]	0.003
— Lymphocytes (10^9/L)	2.3 ± 0.8	1.3 ± 0.7	1.6 ± 1.0	2.0 ± 1.3	0.46
— Monocytes (10^9/L)	0.3 ± 0.1	0.1 ± 0.05	0.3 ± 0.3	0.1 ± 0.2	0.11

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

6

1 **Table II: Fetal liver function tests at delivery.**

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

2 Data are mean ± SD. [a] $p<0.05$ *vs*. control (vehicle only) by one-way ANOVA and post-hoc
3 Tukey's multiple comparison test. Abbreviations: N, group size; AST, aspartate
4 aminotransferase; GGT, gamma glutamyl transpeptidase; GLDH, glutamate dehydrogenase.
5