Aminophylline and progesterone prevent inflammation-induced preterm parturition in the mouse.

Running Title: Aminophylline/progesterone delays LPS parturition

Summary sentence: The combination of aminophylline and progesterone delays LPS-induced parturition and represses inflammation induced gene expression in myometrial explants and cell cultures.

- **Key terms:** pregnancy, progesterone, phosphodiesterases, myometrium, Cyclic adenosine
- monophosphate (cAMP), cytokines

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Abstract

Although progesterone (P4) supplementation is the most widely used therapy for the prevention of preterm labor (PTL), reports of its clinical efficacy have been conflicting. We have previously shown that the anti-inflammatory effects of P4 can be enhanced by increasing intracellular cAMP levels in primary human myometrial cells. Here we have examined whether adding aminophylline (Am), a non-specific phosphodiesterase (PDE) inhibitor that increases intracellular cyclic adenosine monophosphate (cAMP) levels, to P4 might improve its efficacy using *in vivo* and *in vitro* models of PTL.

In a mouse model of lipopolysaccharide (LPS)-induced PTL, we found that the combination of P4 and Am delayed the onset of LPS-induced PTL, while the same dose of P4 and Am alone had no effect. Pup survival was not improved by either agent alone or in combination. Myometrial prolabor and inflammatory cytokine gene expression was reduced, but the reduction was similar in P4 and P4/Am treated mice. There was no effect of the combination of P4 and Am on an *ex vivo* assessment of myometrial contractility. In human myometrial cells and myometrial tissue explants, we found that the combination had marked anti-inflammatory effects, reducing cytokine and *COX-2* mRNA and protein levels to a greater extent than either agent alone.

These data suggest that the combination of P4 and Am has a more potent anti-inflammatory effect than either agent alone and may be an effective combination in women at high-risk of PTL.

Introduction

In developed countries, between 8 and 12.5 % of all births are preterm and they are responsible for more than 85% of all perinatal morbidity and mortality [1]. Preterm labor (PTL) is responsible for 60% of preterm births, but the pathophysiological events that trigger PTL are largely unknown, although many are associated with infection and/or inflammation. In 2011, the FDA approved use of progesterone (P4) supplements to reduce the risk of preterm births in high-risk women with previous history of PTL. However, although P4 has been shown to be effective in some studies of singleton pregnancies at high-risk of PTL, with about a 40% risk reduction [2, 3], in other it has been found to be less effective [4] and to have no effect in multiple pregnancies [5]. Because of the significant mortality and morbidity associated with preterm birth, finding agents, or combinations of agents, which can effectively prevent PTL is a major research goal with significant potential social and economic benefits.

Previously we showed that the combination of P4 and a cyclic adenosine monophosphate (cAMP) agonist reduced the IL-1β-induced increase in cyclooxygenase-2 (COX-2), the inflammation-linked, key enzyme in prostaglandin synthesis [6]. Rolipram, a cAMP specific phosphodiesterase (PDE) inhibitor, has previously been shown to be an effective in delaying the onset of parturition in the lipopolysaccharide (LPS) based mouse model of PTL [7], but the use of rolipram and other PDE4 inhibitors in humans has been associated with marked side effects [8]. Aminophylline (Am), another cAMP-PDE inhibitor, is a xanthine derivative and is known for its use in the treatment of asthma in both pregnant and non-pregnant women. Previously, *in vitro* studies have shown that Am and theophylline promote relaxation in human myometrial tissue [7, 9]. While in women presenting with threatened PTL, Am has been shown to be as effective as antenatal steroids for fetal lung maturation [10] and to reduce the risk of intraventricular haemorrhage in the neonate [11]. In addition, it has been used widely in extremely

preterm infants to reduce the risk of apnoea [12] and bronchopulmonary dysplasia [13] and to improve neonatal outcomes in a similar way to caffeine, another cAMP-PDE inhibitor [14].

In this study, we investigated the effects of the combination of Am, a non-specific PDE inhibitor that elevates cAMP, and P4 treatment both *in vivo*, in a mouse model of inflammation induced PTL, and *in vitro*, in myometrial explants and primary cells. We tested the hypothesis that the combination of P4 and Am is more effective than either agent alone for the prevention of LPS-induced PTL and in the reduction of inflammation induced increases in cytokine and prolabor gene expression.

Material and Methods

In vivo study and sample collection

Animal study ethical statement and husbandry conditions

All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 & European Directive 2010/63/EU, under Home Office License 70/7518 and with approval from Institute Animal Welfare & Ethical Review Boards of Imperial College and University College London. The 3Rs and ARRIVE guidelines were consulted and implemented in completion of this study and in the production of this manuscript.

Female CD1 outbred mice (Charles River, UK) were time-mated, and the day a copulatory plug was observed was designated embryonic day 0 (E0). Mice were group housed (up to 5 dams per cage) in open cages at 21 ± 1°C, on a 12:12 light/dark cycle, fed Harlan Teklad 2018 Global Diet and given water *ad libitum.* The health status of all animals was monitored daily and all mice due to enter a treatment arm were inspected prior to initiation of the protocol. After surgery, mice were singly housed to litter down and monitored by both infra-red CCTV (RF Concepts, Ireland) and physical inspection in the lead up to labor, in case of laboring complications.

LPS-induced preterm labor model and treatment conditions

The mouse model of inflammation-induced PTL is well established in our group [15, 16]. On day 16 of gestation (E16), dams were pre-treated with either an intra-peritoneal injection of vehicle (DMSO; dimethyl sulfoxide, Thermo Fisher Scientific, UK) or 10mg/kg Am (Sigma Aldrich, UK). This was followed 90 minutes later with a subcutaneous injection of vehicle (peanut oil (Sigma Aldrich, UK)) or 5mg P4

(Sigma Aldrich, UK) and after a further 30 minutes, intrauterine (i.u.) injection of vehicle (PBS) or 10µg of *Escherichia coli* LPS serotype 0111:B4 (Sigma Aldrich, UK).

Briefly, the intrauterine injection involved a mini laparotomy performed under isoflurane anaesthesia (5% isoflurane in oxygen at a 1.5L/min flow rate), with Morphine analgesia (5mg/kg). The uterine horns were exteriorised and kept moist with sterile PBS in a sterile swab. Intrauterine injections were performed on the right uterine horn between the first and second uppermost fetuses with care not to enter either amniotic cavity. LPS or sterile PBS (25µl volume) were injected. Each pup was checked for viability before the uterine horns were replaced into the abdomen. Bupivicaine (2mg/kg) was applied to the muscle layer and skin of the abdomen during suturing for further analgesia. Mice were allowed to recover in a heated recovery chamber, followed by single-housing and regular monitoring, prior to culling and tissue collection. Labor time was calculated from the time of injection to the delivery of one or more pups.

Tissue samples were collected from a second group of animals at 7h post LPS injection. Dams were culled via exsanguination by cardiac puncture, followed by immediate cervical dislocation. Pup health status was assessed immediately upon confirmation of the dam's death and prior to tissue collection. Tissue samples from the left uterine horn were snap frozen on dry ice and stored at -80^{o}C.

A third set of mice underwent the same treatment protocol, but uterine horn tissues were stored in ice-cold PBS to be used for *ex vivo* contractility measurements within the same day.

Animals were selected for LPS/control and treatments/controls randomly, with multiple different treatment groups injected alongside each other during every surgical session, to avoid bias. Surgeries

were performed at the same time each experimental session (approximately 1000-1200) to ensure the gestational stage was as similar as possible between mice, thus reducing variation in the model. Intermediate collection time points and sample sizes were determined from our previous studies [15, 16] and to allow for statistical normality testing. We mated enough animals to ensure that we had a minimum of 6 animals at each collection time point for expression studies. Initial laboring studies showed that LPS vehicle control treated animals labored similarly. A statistician was consulted and confirmed that these could be treated as one group with a smaller sample number, so all later figures only present V+V+V (Vehicle) as the single control group even though all treatment combinations were examined but showed no significant differences to the V+V+V group. When the mating proved to be more successful than expected, we used the excess animals to increase numbers in the different LPS treatment groups. A single pregnant dam represents a single experimental unit throughout this study.

Human tissue collection and ethical statement

Study approval was given by the local research ethics committee for Chelsea & Westminster Hospital (London, UK; Ethics No. 10/H0801/45) and samples were obtained in accordance with guidelines set by the Declaration of Helsinki. Informed written consent was obtained from all patients who participated. Biopsies of the myometrium were obtained from the upper margin of the uterine lower segment incision made at the time of Caesarean section from non-laboring women of term (≥37 weeks gestation) singleton pregnancies. Indications for Caesarean section were fetal indication, previous Caesarean section, breech presentation and maternal request. Biopsies were stored in Dulbecco's PBS (DPBS) at 4^{o}C prior to use.

For myometrial tissue explants [17] and cell culture [18], the myometrial biopsies were put into Dulbeccos modified Eagles Medium (DMEM) medium containing L-glutamine and 100 mU/mL penicillin and 100 g/mL streptomycin (Thermo Fisher Scientific, UK) and were stored at 4^{o}C for no more than 3h prior to preparation for explants or cell culture as previously described [17, 18]. The myometrial cells were used between passage 2-4. They were validated by the demonstration of the presence of functional oxytocin receptor [19].

RNA extraction and quantitative RT-PCR

Previously published methods for extraction of RNA and its conversion to cDNA were used for mouse uterine horn tissues [20] and primary human myometrial cells [19]. Quantitative PCR (qPCR) was performed using SYBR Green (Life Technologies) and a Rotor-Gene-Q thermocycler with Rotor-Gene Q Software v2.0.3 (Qiagen). An initial melt step of 10 minutes at 95^{o}C was followed by up to 45 cycles of 95^{o}C for 20 seconds (melt), 58-60^{o}C for 20 seconds (anneal) and 72^{o}C for 20 seconds (elongation), after which 15-second melt steps over the temperature range of 72-99^{o}C ended the qPCR protocol. For each set of primers (Supplementary Tables 1&2), a qPCR standard curve was derived from a 10-fold dilution series of its concentrated PCR product. The qPCR amplification cycle at which SYBR Green fluorescence values showed an exponential increase that was approximately parallel between all samples was identified as the threshold from which data was obtained. All mRNA abundance data for targets of interest were normalised to those of constitutively expressed housekeeping genes: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and/or beta-actin (*ACTB*) for human samples; *Actb,* tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*Ywhaz*) *and* ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit (*Atp5b*)) for mice samples.

Cytokine/chemokine measurements

Mouse myometrial tissue lysates were prepared using a Bio-Plex Cell Lysis kit (Bio-Rad, Hertfordshire, UK) and a Precellys 24 homogeniser (Stretton Scientific) as previously described [21]. Protein concentrations were quantified using a DC (modified Lowry) Protein Assay kit (Bio-Rad). Total mouse myometrial protein lysates (500 µg per sample) were added to the wells of a Bio-Plex Pro Mouse Cytokine/Chemokine Assay multi-well plate. Undiluted media samples from primary human myometrial cells were added to a Bio-Plex Pro Human Cytokine/Chemokine Assay multi-well plate. Cytokine assays were performed according to the manufacturer's instructions, using a Bio-Plex MAGPIX multiplex reader and Bio-Plex Manager v6.1 software (BioRad, Hertfordshire, UK). 500µg of total mouse myometrial protein lysate was added per well to a custom-made Bio-Plex Pro™ Mouse Cytokine Assay (based on previous data) and included the following analytes: IFNγ, IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-12(p40), IL-12(p70), KC, CCL2, MIP-2α, RANTES and TNFα. Undiluted human myometrial cell culture supernatants were analysed using a custom-made Bio-Plex Pro™ Human Cytokine Assay and included the following analytes: IFNγ, IL-1α, IL-1β, IL-6, IL-8, CCL2, CCL11, CCL20, GROa, GROb, ICAM, RANTES and LIF.

Ex vivo isometric tension measurements of mouse myometrial tissue

One uterine horn for each mouse was isolated for dissection into two tissue strips (~15 x 2 mm) after storage in ice-cold PBS for ≤4 h and removal of endometrial layer using a cotton swab. Each myometrial tissue strip was mounted to a MLT0210/D transducer (ADInstruments) connected to a 4-channel organ bath (Panlab) with 4SP PowerLab unit (ADInstruments), and submerged into physiological saline solution (PSS, pH 7.4; 119 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 1.18 mM KH_2PO_4, 25 µM EDTA, 25 mM $NaHCO_3$, 6 mM glucose and 1.6 mM $CaCl_2$) maintained at 37°C with 95 % O_2/5 % CO_2 aeration. Tension (29.4 mN) was applied to all tissues at 5 minutes after initiating data recording and spontaneous

contractions were recorded for 1 h 10 minutes before Syntocinon (synthetic oxytocin; Alliance Pharmaceuticals) was added to tissues to give a final exogenous concentration of 1 nM. After 25 minutes of oxytocin stimulation, tissues were bathed in PSS containing 60 mM KCl (KPSS) for 10 minutes to confirm viability by maximal depolarisation, after which the KPSS was replaced with PSS and tissues were bathed for a further 10 minutes before the experiment was terminated. All contractility data was analysed using LabChart 6 software (ADInstruments) to calculate mean integral tension values as previously described [22]. Spontaneous contractile output was measured from the 30-minute period immediately before oxytocin/H_2O addition to tissues. Hormone-augmented contractile output was measured from last 10-minute period of 25 minutes exposure to 1 nM oxytocin or its H_2O vehicle.

Statistical analysis

Normally distributed data were analysed using a Student t test for two groups and an ANOVA followed by a Dunnett post hoc test for three groups or more. Data that were not normally distributed were analysed using a Mann–Whitney U test for non-paired data and a Wilcoxon matched pairs test for paired data (two groups) and when comparing three groups or more we used a Friedman's Test, with a Dunn's Multiple Comparisons post hoc test. $p<0.05$ was considered statistically significant (PRISM software; GraphPad).

Results

The combination of aminophylline and progesterone abolishes LPS-induced pre-term labor in mice

LPS induced PTL as early as 4.5h post injection, compared to approximately 65-80h in control dams. Neither P4 (5mg) nor Am (10mg/kg) had a significant effect on latency to labor in LPS-treated or control dams. However, the combination of both agents significantly delayed parturition in LPS-treated dams to a mean of 74h, significantly prolonging the latency to labor compared to LPS-treated (9.9h, $p<0.001$), LPS+P4-treated (11.6h, $P<0.01$) and LPS+Am-treated dams (12.9h, $P<0.01$, Figure 1A). The health of pups was assessed at 7h post LPS/PBS administration and in labor. All PBS treated dams had live pups. From dams treated with LPS 40% of pups were alive (although very sickly) at 7h post LPS administration, decreasing to 20% in labor. The survival rates were not improved with P4 alone, Am alone or their combination (Figure 1B&C).

The effect of aminophylline and progesterone on LPS and progesterone responsive genes

LPS responsive genes: Treatment with P4 alone reduced the LPS-induced increased in oxytocin receptor (*Otr*) mRNA levels ($p<0.01$, Figure 2A) and while Am alone had no effect, the combination had a similar effect on *Otr* mRNA levels ($p<0.05$; Figure 2A). *Cox-2* mRNA levels were not affected by either agent alone or in combination (Figure 2B). In terms of pro-inflammatory cytokine mRNA levels, for *Il1β* and *Tnfα*, there was no effect, but for *Il6*, P4 and Am both reduced *Il6* mRNA levels (both $p<0.05$) and the combination had the most consistent effect, but it was no greater than either agent alone ($p<0.01$; Figure 2E). Interestingly, the repression of the mRNA levels was generally not reproduced in the protein levels of myometrial cytokines as assessed 7h post LPS administration (Figure 3). In the case of IL-6, P4

alone reduced levels ($p<0.05$; Figure 3B) and in the case of CXCL1, P4 alone and in combination with Am reduced mRNA levels to a similar extent (both $p<0.01$; Figure 3G).

Progesterone responsive genes: In an earlier study, we identified a series of genes that were increased by IL-1β treatment of human myometrial cells and repressed by P4 [23]. In the current study, we found that P4 did not repress the LPS-induced increase in the mRNA levels of *Il-24, Cxcl2 or Irak3* (Supplementary figure 1 A,B&D), but did repressed *Il-11* mRNA levels ($p<0.001$; Supplementary figure 1 C). In terms of *Cxcl2*, Am and the combination of Am and P4 repressed mRNA levels (both $p<0.05$; Supplementary figure 1 B). And for *Il-11*, Am and the combination of Am and P4 repressed mRNA levels to a similar extent as P4 alone (both $p<0.01$; Supplementary figure 1 C). For *Irak3*, only Am reduced the mRNA levels compared to all other groups ($p<0.05$-0.001; Supplementary figure 1 D). Next, we examined a group of genes that we found to be P4 responsive in human myometrial cells [24]. After LPS administration, of 11beta hydroxysteroid dehydrogenase type 1 (*11bHsd1*), MAP kinase phosphatase 1 (*Mkp1*) and FK506 binding protein 5 (*Fkbp51*), only *FKBP51* mRNA levels increased in response to the combination of P4 and Am compared to LPS alone and LPS/P4 ($p<0.01$ and 0.05 respectively; Supplementary figure 1 G).

Signalling protein activation.

We investigated phosphorylation of various protein kinases and transcriptional factors. At the time point that we monitored, 7h post LPS injection, there were no significant changes in phosphorylation levels of c-Jun N-terminal kinase (JNK), mitogen-activated protein kinases 1&3 (ERK1&2), c-Jun (c-Jun), cAMP response element-binding protein (CREB), heat shock protein (HSP20) or nuclear factor NF-kappa-

B p65 subunit (p65) in response to the treatments (data not shown). Contraction-associated proteins, such as COX-2 and connexin 43, did not change with any treatment (data not shown).

IL1β-induced gene expression in human myometrial cells and tissue.

We used primary myometrial cells and focused on the investigation of two genes *IL-8* and *PTGS2*. Investigating their response over time (6, 24 and 48h) and to different doses of P4 and Am (P4 concentration of 1 and 10μM, and Am of 0.1, 1.0, 10 and 100μM; data for the effects of Am 100μM and P4 10μM are shown in Supplementary figure Fig.2A-F). Our data showed that the effects of Am were similar for all doses, so we went on to use 0.1 and 10μM in combination with 1 and 10μM of P4 at 24h. The effects of P4 alone, Am alone and in combination on the levels of IL-1β, IL-6, TNFα, CXCL1 and CXCL2, were studied at low (Am 0.1 and P4 1.0μM) and high (Am 10 and P4 10μM) doses (Fig.4A-L). We found that in the case of IL-1β at both low and high doses P4 and Am alone reduced IL-1β levels and the effect of the combination was greater (Fig4A&B). For IL-6, TNFα and CXCL1 at both high and low doses, P4 and Am alone had no effect, but the combination of P4 and Am reduced the level of each analyte vs. all other groups (Fig.4C-J). This was true for CXCL2 as well, except for high dose Am, which reduced CXCL2 levels (Fig.4J). For IFNγ, there was no effect of either P4 or Am or their combination on the IL-1β-induced increase (Fig.4K&L).

In myometrial explants, we found that both P4 alone and the combination of P4 and Am reduced the IL-1β-induced increase in COX-2 protein levels ($p<0.01$ and 0.05 respectively; Figure 5).

Ex vivo myometrial contractility was not altered by aminophylline and P4

The spontaneous and oxytocin-augmented contractility of myometrial tissue isolated from mice after treatment with P4 and Am, or their vehicle controls, was measured by *ex vivo* isometric tension measurements to evaluate their physiological impact in the absence of LPS exposure. From which, we found no difference in contractile output, represented by MIT, for both types of contractions when these two *in vivo* treatments were compared (Supplementary Figure 3).

Discussion

In this study, we found that the combination of P4 and Am treatment delayed the onset of i.u. LPS-induced PTL in the mouse. The combination of P4 and Am did not improve neonatal outcomes, but the use of Am was not associated with any adverse maternal side effects compared to our observations with rolipram or milrinone (unpublished observations). Intriguingly, the effect of the combination of P4 and Am on mouse myometrial cytokine and prolabor mRNA levels was similar to P4 alone, questioning the mechanism underpinning the beneficial effect of the combination. In human myometrial cells, we found that the combination of P4 and Am had a marked effect on the IL-1β-induced increases in pro-inflammatory mediators, which was significantly greater than either agent alone.

Previous studies have shown that some P4 formulations, including natural P4 [25] and synthetic progestogens, medoxyprogesterone acetate (MPA) and 17 alpha hydroxyprogesterone caproate (17OHPC), can repress LPS-induced PTL in the mouse [26, 27], which, in the case of 17OHPC, was associated with a significant maternal mortality [27]. Here we observed that P4 was well tolerated, but that at the dose we used, it had no effect on rates of PTL, although it did repress mRNA levels of some prolabor and pro-inflammatory factors. Similarly, other groups have reported that rolipram, the type 4

phosphodiesterase inhibitor, can effectively delay LPS-induced PTL [7], but its use in humans is associated with side effects. In pregnant mice, we found that rolipram had side effects (unpublished observation); but, in contrast, Am 10mg/kg was well tolerated. Equally, at this dose Am did not delay LPS-induced PTL and had no effect on myometrial prolabor and pro-inflammatory factors. Only when Am was used in combination with P4 did we see a significant delay in LPS-induced PTL; however, although PTL was prevented, we did not observe any greater effect on inflammatory cytokine or prolabor mRNA levels, suggesting that other mechanisms may be responsible. We assessed myometrial contractility *ex vivo,* after treatment with Am and P4, but found no evidence of a reduction in spontaneous or oxytocin-induced myometrial contractility. This may suggest that the effect is not mediated via the contractile apparatus or that any effect on contractility is only revealed after treatment with LPS, which was not assessed in this study.

We found that Am was well tolerated by the mice in our study at the dosage used, but in humans, Am can cause tachycardia and palpitations, tremor and gastrointestinal upset. Equally, in an ongoing feasibility study on the use of Am in pregnant women at high risk of PTL, we found that it was well tolerated (unpublished observation). Similarly, although the beneficial effects of the combination of P4 and Am in the mouse were reproduced in human tissues and cells, the hormonal control of pregnancy is very different in the mouse compared to the human and the model of LPS-induced PTL in the mouse is limited in its ability to reproduce human PTL. Consequently, whether the beneficial effects found in this study can be reproduced in the human remains to be confirmed.

The failure to improve pup survival may mean that the inflammatory process is not completely repressed by the combination of Am and P4, such that it is still able to induce fetal demise. However, LPS-induced pup mortality may be mediated through a reduction in placental perfusion, as LPS treatment in pregnant rats results in a cessation of placental blood flow [28]. This may be secondary to

hypotension, but although high-dose P4 prevents the hypotensive response to LPS and delays the onset of LPS-induced PTL, it also had no effect on pup survival [25] , suggesting that hypotension is not directly responsible for pup demise in the mouse. Only in our studies involving CCR2-/- mice did we see a delay in pup death, implying that this is a cell-mediated, or at least modulated, process (Hua et al, unpublished observation). Consequently, the absence of improved pup survival could imply that immune cell activation is not reduced by the combination of P4 and Am. The effect of the combination of P4 and Am on maternal blood pressure and immune cell activation awaits more detailed study.

Our current data support our earlier observations made in a series of *in vitro* experiments using human myometrial cells, in which we found that the combination of a cAMP agonist and P4 enhanced the ability of P4 to repress IL-1β-driven COX-2 expression, p65 nuclear translocation and DNA binding [6] . In addition, cAMP enhanced P4 transcriptional activity by increasing P4/progesterone receptor (PR)-B DNA binding and Progesterone response element (PRE) activation, while at the same time increasing PR-B levels, so reducing the PR-A:PR-B ratio and reducing the association between PR and Nuclear Receptor Corepressor [6], a negative co-regulator of PR-action [6]. We also showed that the combination of cAMP and P4 enhanced the P4-induced increase in FKBP51 mRNA levels [6] . In the current study, we assessed *Fkbp51*mRNA levels in mouse myometrium and found that the combination of P4 and Am increased *Fkbp51* mRNA expression over that observed by LPS and the combination of LPS and P4, suggesting that *in vivo*, as well as *in vitro* cAMP agonists enhance the transcriptional activity of P4 perhaps accounting for the beneficial effect of the combination over P4 alone. In a further study, we found that the combination of P4 and a cAMP agonist repressed inflammation-induced prolabour gene expression via PKA and, intriguingly, following knockdown or inhibition of PKA, the combination of P4 and a cAMP agonist actually enhanced inflammation-induced prolabour gene expression (unpublished observation).

We found no effect of P4 and Am alone or in combination on the level of phosphorylation of various protein kinases, transcription factors in mouse myometrium. These data may be because the changes in phosphorylation occurred earlier than the 7h time point that we used. We also found no differential effects on total or phospho HSP20 protein levels (data not shown). HSP20 can be phosphorylated by both cAMP- and cGMP-dependent protein kinases (PKA/PKG) and the increase in phosphorylation is associated with smooth muscle relaxation [29]. However, the lack of change in both HSP20 levels (or phosphorylation) suggest that the P4/Am combination does not work through modulation of HSP20 function. In addition, we found that changes in mRNA and protein levels were not always consistent although the trends were similar, this has been observed in other studies and may reflect the temporal differences in that mRNA is expressed and then translated into protein and the fact that other factors, such as siRNA, may block translation preventing any increase in mRNA being reflected at the protein level. Similarly, changes in protein metabolism may mean that protein levels change independent of changes in mRNA.

We recently described the use of myometrial explants for the study of whole myometrial tissue as a means of more accurately reproducing the *in vivo* situation in the laboratory [17]. Here, similar to our human myometrial cell data, we found that P4 alone and the combination of P4 and Am repressed the IL-1β-induced increase in COX-2 protein. These results contrast to our *in vivo* mouse data, where P4 and Am alone or in combination failed to repress the LPS-induced increase in myometrial COX-2 protein levels. The differences may reflect a species difference or that our sampling time points missed any effect of P4 and/or Am on myometrial COX-2 levels.

These data provide preliminary evidence that the addition of Am might enhance the ability of P4 to delay PTL in high-risk women. To study this, our group is currently undertaking a clinical trial investigating the efficacy of Am and P4 combination therapy in the prevention of PTL in humans. To date

there have been multiple term deliveries of live and well babies to women on the combined treatment who previously delivered prematurely.

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Figure Legends

Figure 1

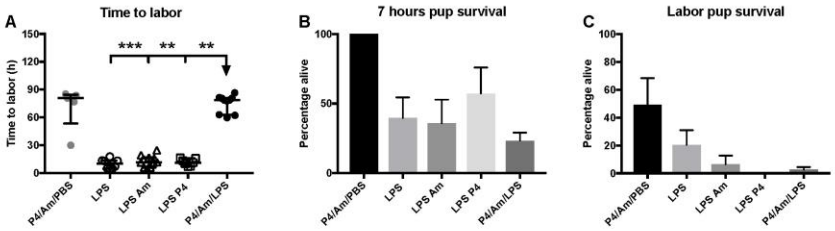

Figure 1 Latency to labor post LPS injection (A). Pregnant female CD1 mice were treated with vehicle or aminophylline ip at 2h and/or progesterone sc at 30 minutes prior to intrauterine injection of either PBS or LPS. The time taken to labor post LPS injection was noted. Data are shown as a scatter plot with median and interquartile range superimposed with control samples for illustration only and were analysed using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test **p<0.01, ***p<0.001; n=10-14 for each LPS group and n=5 for each PBS group. **Pup survival at 7h post LPS injection and in labor (B&C).** Individual pups were examined at the time of tissue collection, from a second group of dams for each of the treatment groups at 7h post-LPS injection and at delivery. Pups were defined as alive or dead, n= a minimum of 6 litters per group. Data are shown as mean and SEM.

Figure 2

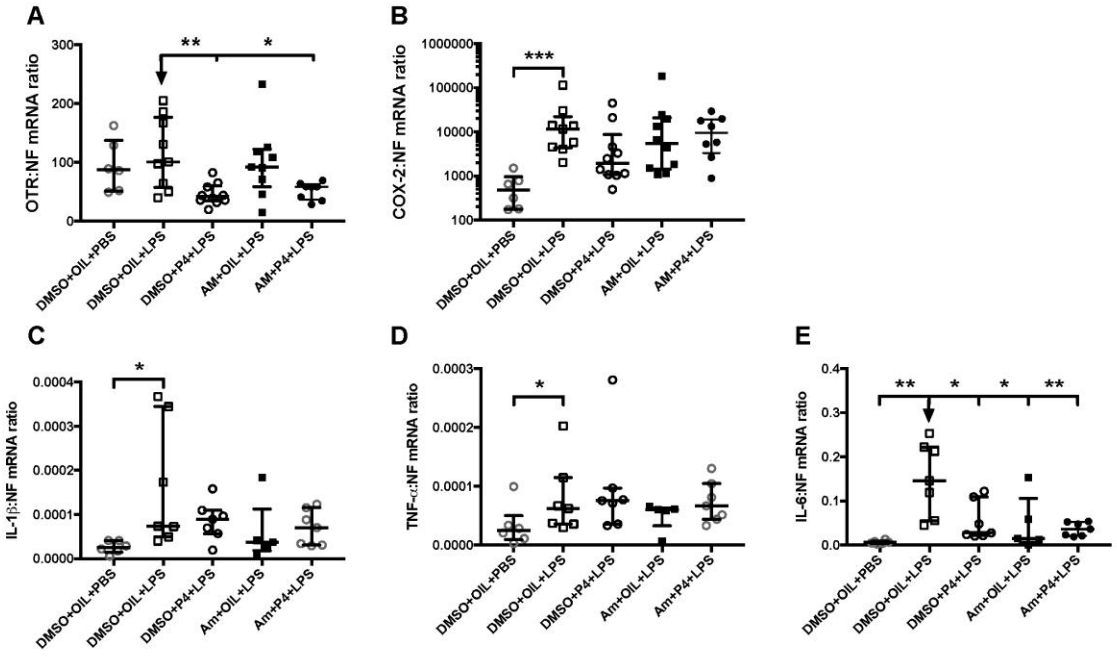

Figure 2 (A-E) Effects of aminophylline and progesterone on LPS responsive genes expression levels. Myometrial mRNA expression levels of several LPS responsive genes thought to be important in labor, including (A) OTR, (B) PTGS2, (C) IL-1β, (D) TNFα and (E) IL-6 were analysed by rt-PCR from samples collected 7h post-LPS administration. mRNA levels are shown here as a ratio of expression level relative to a normalisation factor calculated from 3 housekeeping genes. Data are shown as a scatter plot with median and interquartile range superimposed and were analysed using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed, and using ANOVA, with Dunnett and Bonferroni's post-test for data that were normally distributed *p<0.05, ** p<0.01, ***p<0.001 (n=6-8 per group).

Figure 3

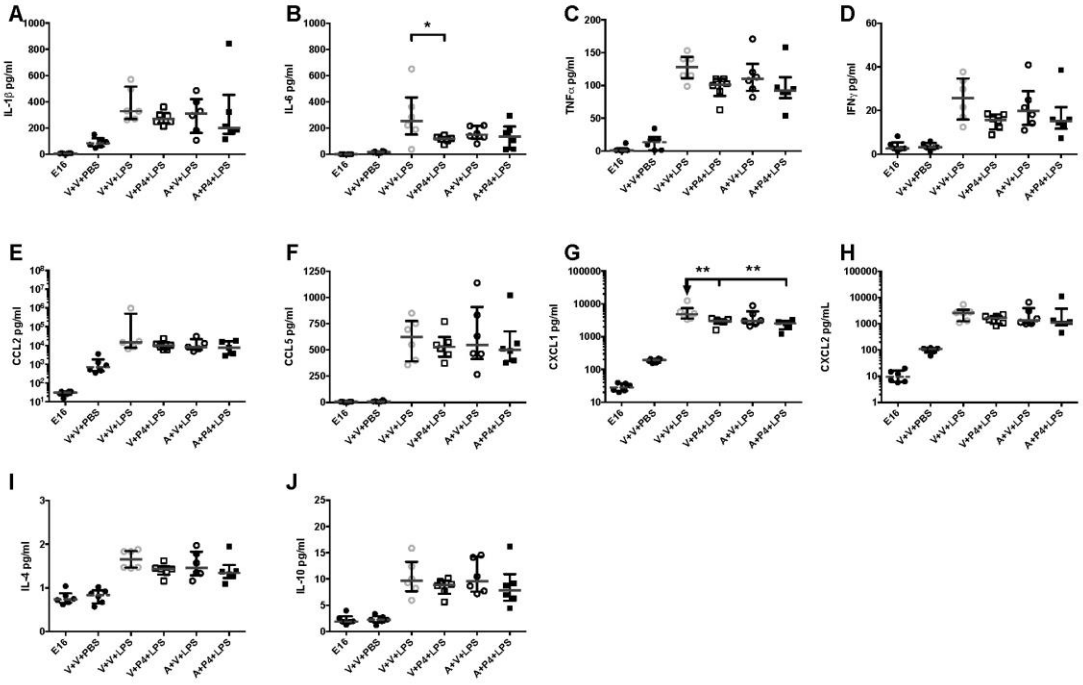

Figure 3 (A-J) Effects of aminophylline and progesterone on murine myometrial inflammatory cytokine levels. Myometrial protein lysates were analysed on a Bioplex Pro-Cytokine assay to look at the effect of the different treatment groups on LPS-induced inflammatory markers. Cytokines examined include: (A) IL-1β, (B) IL-6, (C) TNFα, (D) IFNγ, (E) CCL2, (F) CCL5, (G) CXCL1, (H) CXCL2, (I) IL-4, (J) IL-10; Data are shown as a scatter plot with median and interquartile range superimposed with control samples for illustration only and were analysed using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed, and using ANOVA, with Dunnett and Bonferroni's post-test for data that were normally distributed n=6-8 per group; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

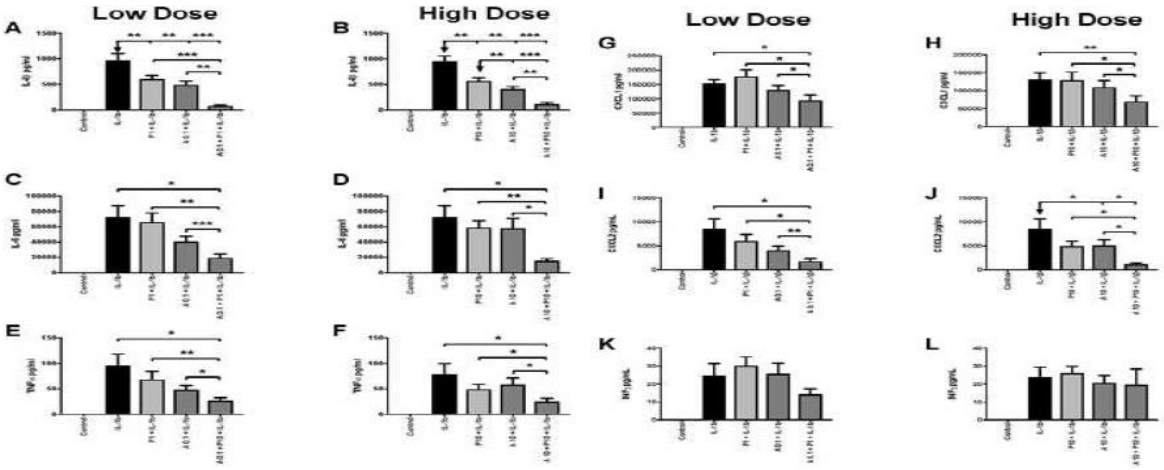

Figure 4 Effects of the combination of low-dose and high-dose aminophylline and progesterone treatment on IL-1β stimulated human myometrial cell supernatant cytokines levels. Human myometrial cells were isolated from myometrial biopsies obtained from women at the time of pre-labor Caesarean section. Cells were cultured and incubated with IL-1β (10ng/mL) with or without progesterone (1 μM) and aminophylline 0.1μM alone and in combination (low-dose) and aminophylline 10 μM with or without progesterone (10μM) alone and in combination (high-dose) for 24h. The levels of cytokines were measured using a Multiplex assay. Data are shown as the mean and SEM with control samples for illustration only, and were compared using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test as the data were not normally distributed, and using ANOVA, with Dunnett and Bonferroni's post-test for data that were normally distributed *p<0.05, **p<0.01 (n=7-9 in each experiment).

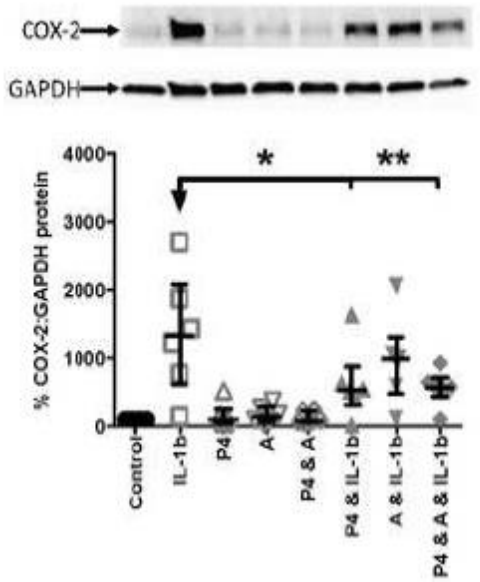

Figure 5 Effects of aminophylline and progesterone treatment on IL-1β stimulated human myometrial tissue explants on COX-2 protein levels. Human myometrium obtained from non-laboring women was finely dissected into 3x3x3mm^3 explants. Explants were immediately pre-treated for 6h with ethanol vehicle, 1μM P4, 10μM aminophylline either alone or in combination followed by a 24h treatment with IL-1β (10ng/mL). Explants were subsequently snap frozen in liquid nitrogen. Protein was extracted and quantified with Western blotting for COX-2 as described in Materials and Methods. Data are shown as a scatter plot with median and interquartile range superimposed with control for illustration only and were analysed using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test as the data were not normally distributed * $p<0.05$; ** $p<0.01$; (n=6-8).

Supplementary Legends

Supplementary Figure 1 Effects of aminophylline and progesterone on progesterone responsive gene expression levels. Two groups of genes were studied, the first a group of genes which were increased by IL-1β in human myometrial cells and which were repressed by progesterone, (A) IL-24, (B) CXCL2, (C) IL-11 and (D) IRAK-3, and a second group of genes whose expression was increased by progesterone in human myometrial cells (E) FKBP51, (F) MKP-1 and (G) 11βHSD1. These genes were analysed in mouse myometrium exposed to LPS alone or in combination with progesterone, aminophylline or their combination. The samples were analysed by rt-PCR from samples collected 7h post-LPS administration. The mRNA levels shown here as a ratio of expression level relative to a normalisation factor calculated from 3 housekeeping genes. Data are shown as a scatter plot with median and interquartile range superimposed and were analysed using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed, and using ANOVA, with Dunnett and Bonferroni's post-test for data that were normally distributed *$p<0.05$, ** $p<0.01$, ***$p<0.001$ (n=6-8 per group).

Supplementary Figure 2 Effects of aminophylline and progesterone treatment on IL-1β stimulated human myometrial cells on COX-2 and IL-8 mRNA expression at 6, 24 and 48h time point. Human myometrial cells were isolated from myometrial biopsies obtained from women at the time of pre-labor Caesarean section. Cells were cultured and incubated with IL-1β (10ng/mL) alone or with progesterone 10μM alone or with aminophylline 100μM or their combination for 6, 24 or 48h. The levels COX-2 mRNA (A) and IL-8 mRNA (B) were measured using rt-PCR. Data are shown as the mean and SEM with control for illustration only and were analysed using Friedman's Test, with a Dunn's Multiple Comparisons post hoc test for data that were not normally distributed, and using ANOVA, with Dunnett and Bonferroni's

post-test for data that were normally distributed *p<0.05, **p<0.01, *** p<0.001 (n=7-9 in each experiment).

Supplementary Figure 3 Spontaneous and oxytocin-augmented *ex vivo* contractility of mice myometrial tissue after 9h *in vivo* treatment with aminophylline and progesterone. Pregnant female CD1 mice were injected (intra-peritoneal) with aminophylline (10 mg/kg) or its phosphate-buffered saline (PBS) vehicle for 90-minute treatment, followed by injection (subcutaneous) with progesterone (5 mg/kg) or its peanut oil vehicle for 7.5h treatment prior to culling for isolation of uterine horn tissues. One uterine horn from each mouse was dissected into 2 tissue strips, after removing endometrial layer, for isometric tension measurements. For each experiment, both tissue strips were recorded for 1h 10min of spontaneous contractions before they were treated with either oxytocin (1nM) or H_2O for 25 minutes, after which tissues were stimulated with PSS that contained 60 mM KCl (KPSS) for 10 minutes to confirm viability and subsequently washed with PSS (4.7mM KCl) for 10 minutes before data recordings were terminated. Spontaneous contractile output was measured from the 30-minute period immediately before oxytocin/H_2O addition to tissues. Oxytocin/H_2O-associated contractile output was measured from last 10-minute period of 25 minutes exposure to 1nM oxytocin or its H_2O vehicle. MIT data represented as mean ± SEM and were assessed using Mann-Whitney *U* test for 'before *versus* after' oxytocin/H_2O treatment comparisons for each 9h *in vivo* treatment (* $p \leq 0.05$, ** $p \leq 0.01$). Representative traces of isometric tension measurements are also shown, where time period of stimulation with oxytocin or its vehicle (H_2O) is indicated by the labelled horizontal line above each trace.