Maternal Azithromycin Therapy for *Ureaplasma* Intra-Amniotic Infection Delays Preterm Delivery and Reduces Fetal Lung Injury in a Primate Model

Peta L. Grigsby, PhD[1], **Miles J. Novy, MD**[1], **Drew W. Sadowsky, PhD**[1], **Terry K. Morgan, MD.,PhD**[2], **Mary Long, PhD**[3], **Ed Acosta, PharmD**[3], **Lynn B Duffy, MT**[4], and **Ken B. Waites, MD**[4]

[1]Division of Reproductive & Developmental Sciences, Oregon National Primate Research Center, Beaverton, OR

[2]Department of Pathology, Oregon Health & Science University, Portland, OR

[3]Department of Pharmacology and Toxicology, University of Alabama at Birmingham, AL

[4]Department of Pathology, University of Alabama at Birmingham, AL

Abstract

Objective—We assessed the efficacy of a maternal multi–dose azithromycin (AZI) regimen, with and without anti–inflammatory agents to delay preterm birth and to mitigate fetal lung injury associated with *Ureaplasma parvum* intra–amniotic infection (IAI).

Study Design—Long–term catheterized rhesus monkeys (n=16) received intra–amniotic inoculation of *U. parvum* (10^7 CFU/ml, *serovar 1*). After contraction onset, rhesus monkeys received either no treatment (n=6); AZI (12.5mg/kg, q12h, IV for 10 days; n=5); or AZI *plus* dexamethasone (DEX) and indomethacin (INDO; n=5). Outcomes included amniotic fluid pro–inflammatory mediators, *U. parvum* cultures & PCR, AZI pharmacokinetics and the extent of fetal lung inflammation.

Results—Maternal AZI therapy eradicated *U. parvum* IAI from the amniotic fluid within 4 days. Placenta and fetal tissues were 90% culture negative at delivery. AZI therapy significantly delayed preterm delivery and prevented advanced fetal lung injury, although residual acute chorioamnionitis persisted.

Conclusions—Specific maternal antibiotic therapy can eradicate *U. parvum* from the amniotic fluid and key fetal organs, with subsequent prolongation of pregnancy which provides a therapeutic window of opportunity to effectively reduce the severity of fetal lung injury.

Key Words/Phrases

Antenatal antibiotic therapy; Bronchopulmonary dysplasia/Chronic Lung Disease; Fetal brain injury; Preterm birth; Chorioamnionitis and Mycoplasmas

Corresponding Author:*Peta L. Grigsby, Ph.D.*, Oregon National Primate Research Center (ONPRC), Division of Reproductive & Developmental Sciences, 505 NW 185th Ave, Beaverton, OR 97006, Tel: 503–690–5286, Fax: 503–690–5515, grigsbyp@ohsu.edu.

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INTRODUCTION

Maternal genital tract infections are an important and potentially preventable cause of preterm birth, especially the birth of the most immature infants.[1–3] Among bacterial pathogens, genital mycoplasmas play a unique role in causing or contributing to adverse obstetrical outcomes at virtually every stage of pregnancy.[4, 5] Isolation of *Ureaplasma* species from the placental membranes and amniotic fluid has been consistently associated with histologic chorioamnionitis, preterm birth, and adverse perinatal outcomes.[2, 4, 6–10] *Ureaplasma* spp. are the most common microorganisms isolated from the amniotic fluid, cord blood, respiratory tract, and cerebrospinal fluid (CSF) of infants born prematurely who develop chronic lung disease/bronchopulmonary dysplasia (BPD) and neurodevelopmental disabilities.[2, 6, 11, 12] There is compelling evidence from human studies and experimental models that *in utero* infection with *Ureaplasma* spp. elicits a sustained and dysregulated immune response which is a harbinger of the BPD phenotype.[7, 13, 14]

We have previously demonstrated in a rhesus monkey model that *U. parvum* as the sole pathogen in the amniotic cavity elicits a robust inflammatory response associated with chorioamnionitis, a progressive increase in uterine activity culminating in preterm labor and delivery and fetal lung injury.[7] Similarly, there are clinical reports indicating that *Ureaplasma* infections during pregnancy elicit intense inflammatory responses in the fetus, amniotic cavity and the mother.[15–23] The extent of the inflammatory response and severity of fetal lung damage (progressive alveolitis and bronchiolitis) were related to the duration of *in utero* infection.[7] Intra–amniotic *Ureaplasma* infection alters fetal lung development and augments a pro–inflammatory, pro–fibrotic response in the preterm lung exposed postnatally to ventilation and hyperoxia.[4, 13] Despite *in vitro* susceptibility of *Ureaplasma* spp. to erythromycin, trials of erythromycin therapy in the first four weeks of neonatal life in *Ureaplasma* colonized preterm infants fail to demonstrate efficacy in preventing BPD or eliminating respiratory tract colonization.[4, 13, 24] To prevent chronic lung disease /BPD it may be necessary to eradicate *Ureaplasma* spp. with antenatal antibiotic therapy in order to forestall the additive effects of fetal lung injury with postnatal ventilation and/or high oxygen exposure. Although there are reports of maternally administered erythromycin for the eradication of IAI, there is little agreement in the literature regarding the effectiveness of erythromycin to prevent preterm delivery or eliminate intra–amniotic *Ureaplasma* infections during pregnancy.[25–29]

Despite the large body of evidence which links maternal subclinical infection with premature labor, there is controversy whether antenatal antibiotic intervention inhibits preterm labor, prolongs gestation or otherwise improves perinatal outcomes.[30–33] A prevailing attitude of therapeutic nihilism regarding the role of antibiotics in the prevention of prematurity or neonatal sequelae maybe unwarranted given that conflicting results of these clinical trials may reflect shortcomings and/or variations in study design.[4, 34] Among confounders, are the inclusion of women with preterm contractions without infection, administration of antibiotics at varying stages of intra-uterine infections, and antibiotics that do not target the appropriate pathogen. Alternatively antibiotics alone without the addition of anti–inflammatory agents may not effectively treat chorioamnionitis or reduce the pro–inflammatory mediators that play a key role in the initiation of labor.[4, 35] There is now a critical need to study specific antibiotic and anti–inflammatory regimens for defined pathogens under experimental conditions to evaluate placental transfer and pharmacokinetics (PK) and to establish biological plausibility for antenatal treatment of *Ureaplasma* infections *in utero*.

The primary objectives of the current study were to assess the efficacy of antenatal azithromycin (AZI) in the treatment of experimental intra–amniotic infection (IAI) with *U. parvum* and in mitigating the extent of fetal lung injury *in utero*. Azithromycin is a 15–member azalide, a subclass of macrolide antibiotics with structural similarity to erythromycin, but with a prolonged duration of action, improved tissue penetration and extended range of coverage against *Ureaplasma* spp.[36] We compared *U.parvum* IAI alone (untreated animals) with the effects of maternal AZI treatment alone or in combination with the anti–inflammatory drugs, dexamethasone (DEX) and indomethacin (INDO) on eradicating *U. parvum* IAI, modulating pro–inflammatory mediators, inhibiting preterm uterine activity, delaying premature birth and ameliorating fetal lung injury.

MATERIALS AND METHODS

Animal Model

Study protocols were approved by the Institutional Animal Care and Utilization Committee and guidelines for humane care were followed. Timed–pregnant rhesus monkeys (*Macaca mulatta,* n=23) were adapted to a vest and mobile catheter protection device.[34] Intrauterine surgery was then performed at 119 days of gestation (range: 115–127 dGA) to implant fetal ECG electrodes, and catheters in the amniotic fluid, maternal femoral vein and artery.[34] Intravascular catheters were implanted in the fetal jugular vein, and carotid artery in a select number of fetuses (n=2) for the analysis of AZI concentrations in the fetal circulation following maternal treatment. Post–operative infusions of cefazolin sodium to prevent infection and tocolytic medications to control uterine irritability (e.g., terbutaline sulfate and/or atosiban) were prescribed as previously published.[34] Tocolytic medications were discontinued when uterine quiescence was achieved and at least 48h before experimental procedures.

Microorganism

U. parvum (serovar 1) a low passaged clinical isolate was originally recovered in pure culture from the placenta of a woman with chorioamnionitis who delivered a preterm infant with *Ureaplasma* sepsis.[5] The isolate was initially serotyped by immunoblotting with monoclonal antibodies and subsequently the *Ureaplasma* species and serovar was determined by by real–time PCR.[37] The organisms were grown in 10B broth, frozen, and stored in aliquots at −80°C until utilized for experimental protocols. Inoculum sizes were determined by assessing the number of colony forming units (CFU/ml) in thawed samples and making the appropriate dilutions to deliver the desired number of organisms.[38]

Experimental Design

Animals were allocated to the study by random assignment from the ONPRC colony and treatment and control (IAI untreated) were performed concurrently. Intra–amniotic inoculation was performed on approximately day 128 of gestation (range: 124–135 days; n=16) with *U. parvum* $7–14 \times 10^7$ (CFU/ml). A subset of animals then received either, maternal AZI treatment alone (12.5/mg/kg, q12h, IV for 10 days; n=5) or in combination with DEX (4mg/kg/day, IV for 4 days) and INDO (100mg/day, PO for 5 days; n=5). Antenatal antibiotic/anti–inflammatory treatments commenced after 6–8 days of *U. parvum* IAI and with a concomitant increase in uterine activity and/or cervical effacement/dilation over a 24h observational period, as determined by a modified Bishop's score. The modified Bishop's score assessed the following four components upon vaginal examination; cervical consistency, cervical length, cervical dilation, fetal station. Each component was scored 0–3, with a maximum score of 12. A modest increase in uterine activity (3000–5000 HCA) and/ or a Bishop's score of >5 was utilized as an indicator for the commencement of treatment. Primary comparisons were made between the AZI treatment groups and IAI untreated

animals, which served as treatment 'controls'. Additional historical controls were included for comparative evaluation of placental and fetal lung histopathology between *U.parvum* IAI untreated and antimicrobial treatment groups. These control animals received either a single intra-amniotic bolus infusion of sterile sucrose phosphate buffer (n=3) or physiologic saline (n=4) in place of *U.parvum* on day 135 of gestation (range: 131–138 days).[34] Post–study antibiotic treatment (Zithromax, 500mg, PO for 1 day; 250mg, PO for 4 days) was administered to ensure clearance of the organisms from each animal prior to its return to the colony.

Sampling Protocol for Azithromycin Pharmacokinetics

Following the *initial* dose of maternal AZI, an intensive sampling protocol was followed in which paired samples were taken from maternal plasma and amniotic fluid at 0 (to indicate a trough sample before infusion) 0.5, 1, 2, 3, 4, 6, 8, 12 and 24h, then every 12h thereafter during 10 days of maternal AZI administration. The intensive sampling protocol was repeated after the *final* dose of AZI and sampling was continued periodically throughout the washout phase to compare maternal plasma and amniotic fluid AZI concentrations with results from the *initial* sampling period. In order to assess the placental transfer of AZI to the fetal circulation samples of fetal plasma were obtained from two animals during the *initial* maternal AZI dosing and subsequently all fetal cord blood samples were paired with maternal plasma samples at delivery. Transfer between maternal and fetal compartments was estimated using the percentage of maternal plasma concentrations. Pharmacokinetic analyses were performed by a two-compartment model (forced function, ADAPT II) in order to determine the rate of transplacental transfer into the amniotic fluid compartment and $t_{1/2}$ life as previously described.[39]

Uterine Activity, Preterm Labor and Cesarean Section

Intra–amniotic pressure was continuously recorded from the time of surgery, digitized and analyzed as previously described,[40] with the addition of a new data acquisition system (PowerLab® ADInstruments, ML880 16/30P; LabChartPro™ software v6.1.2). The integrated area under the intrauterine pressure curve was used as the measure of uterine activity and reported as the mean of the cumulative hourly contraction area (HCA; mmHg.sec/h) over 24h. Preterm labor was defined as HCA >8000 (mmHg.sec/h) for more than two consecutive hour epochs and with associated changes in cervical effacement, dilation (Modified Bishop's score >8, determined by vaginal exam over a 24h observational period) or preterm premature rupture of membranes. Cesarean section was performed to optimize the collection of intact gestational tissues when vaginal delivery was considered imminent (as defined above).

Amniotic Fluid Cytokines, Prostaglandin E$_2$ and F$_{2\alpha}$ and Leukocytes

Beginning 48h before IAI, amniotic fluid was sampled daily until delivery. Samples were centrifuged and the supernatant frozen and stored at −20°C as previously described.[40] Quantities of interleukin (IL) –1β, IL–6, IL–8, TNF–α, PGE$_2$, and PGF$_{2\alpha}$ were determined using commercially available human or rhesus monkey specific ELISA assay kits (BioSource International, Camarillo, CA) and enzyme immunoassay (EIA) kits (Cayman Chemical, Ann Arbor, MI) previously validated for use in the rhesus monkey.[7, 41] Amniotic fluid leukocytes from centrifuged cell pellets were counted daily using a hemocytometer after erythrocyte lysis in 2% glacial acetic acid.

Azithromycin (AZI) Concentrations

AZI (ng/ml) was quantified from 100μL of plasma or amniotic fluid using a liquid–liquid extraction method coupled with high performance liquid chromatography tandem mass

spectrometry (HPLC–MS–MS) detection.[39, 42] A standard curve was prepared in either plasma or amniotic fluid with AZI concentrations (range: 6–3000ng/ml). Quality control samples were prepared by spiking plasma with AZI for final concentrations of 18, 180, and 1800ng/ml and erythromycin (internal standard, 100ng/ml). AZI was extracted from all unknown samples, standards and quality control samples by addition of 2ml of methyl–tert–butyl ether. Separation of mobile phase analytes was achieved using reverse–phase HPLC with an isocratic run on an XTerra C8 column (Waters Corp., Milford, MA). Acceptance criteria for the assay mandated that the back–calculated values for curve and quality control standards were within ±15% of nominal concentration. All inter– and intra–coefficients of variation were <10%.

Culture & PCR Analysis

Quantitative cultures and PCR for *U. parvum* were performed on serial samples of amniotic fluid and maternal whole blood obtained *before* and *after* inoculation, and on fetal cord blood at cesarean section delivery. PCR and cultures were also performed on fetal cerebrospinal fluid (CSF), pieces of the lower right lobe of the lung, fetal brain, fetal membranes and placenta. Fetal membrane culture swabs were collected as described previously.[7] To ensure each animal was free of natural mycoplasma infections *before* inoculation, and to guarantee clearance of *U. parvum* from each animal prior to its return to the colony, maternal nasal and vaginal swabs for PCR and culture for genital mycoplasmas were taken at pre– and post–study time points. Periodic amniotic fluid cultures were obtained for facultative and anaerobic bacteria using standard bacteriological procedures.

Samples of fluids, blood, and tissues were frozen immediately upon collection at −80°C until transported on dry ice to the Diagnostic Mycoplasma Laboratory at the University of Alabama at Birmingham. Once received, specimens were processed quantitatively using 10B broth media and A8 agar as described previously.[38] *U. parvum* colonies were identified on A8 agar by urease production in the presence of $CaCl_2$ indicator. Real–time PCR assays were performed on batched specimens containing all samples from a single animal using primers as described previously by Xiao *et al.*[37]

Histopathology

After cesarean section, fetuses were euthanized by barbiturate overdose followed by exsanguination and necropsy performed for tissue collection. Fetuses and placentas were examined, weighed, and evaluated for abnormalities against gestational age–matched controls. Fetal and maternal surfaces of placenta and chorioamnion were assessed for integrity, clarity, coloration and infarction. Fetal tissues were collected as previously described,[7] fixed in neutral buffered formaldehyde, embedded in paraffin, stained with hematoxylin and eosin (H&E) and subsequently sectioned at 5μm for standard histologic examination by a trained pathologist (TKM).

Histologic Evaluation of the Fetal Lung and Placental Membranes

Fetal lung H&E tissue slides were reviewed by three investigators blinded to the experimental conditions. Microscopic lung lesions were semi–quantitatively scored from 0 to 3, for the presence, and extent of alveolar macrophages, neutrophils and lymphocytic infiltration of alveolar walls, using a modified method from Viscardi *et al.*[43] The scoring was determined as, 0 = none; 1 = the presence of polymorphonuclear cells and mild lymphocytic infiltration of alveolar walls; 2 = focal increases in both mononuclear and polymorphonuclear cells in alveoli; 3 = extensive inflammation in both alveoli and terminal bronchioles, exuberant proliferation and sloughing of type II pneumocytes, diffuse interstitial collections of mononuclear cells with multiple peribronchiolar lymphocytic aggregates and hyperplasia of the overlying epithelium.

Multiple representative areas of the gross placenta and fetal membrane were sampled for histologic examination and scored for signs of IAI, defined as the presence of neutrophils marginating into the membranes as described by Romero *et al.*[44] A *'negative'* score indicated no neutrophils present.

Statistical Analysis

Data are presented as Mean ± SEM. Data were first examined for normality by the Kolmogorov–Smirnov test and the Levene Median test for equal variance, and where necessary, data were transformed by natural logarithm to equalize variance. Outcomes of selected gestational ages (i.e., at *surgery*, *inoculation* and *delivery*) and experimental intervals (i.e., from *inoculation–to–treatment*, *to–delivery*, *to–peak* amniotic fluid *U. parvum* colony counts) were analyzed by 1–way analysis of variance (ANOVA) followed by Tukey's all pairwise comparison test. The intervals from *time–to–zero* (CFU/ml) following the onset of AZI treatment were compared by t–test. For statistical analysis of uterine activity, the mean cumulative 24h HCA (mmHg.sec/h) was used and compared by 1–way ANOVA between all experimental groups (IAI alone, AZI alone and AZI *plus* DEX/INDO groups). Similarly, amniotic fluid pro–inflammatory mediators (PGE_2 and $F_{2\alpha}$, cytokines) and leukocytes, were statistically compared using 1–way ANOVA on repeated measures, followed by Tukey's test for all pairwise comparisons on combined baseline values before inoculation (n=16) versus combined peak concentrations during IAI (n=16). Minimum values during AZI alone or AZI *plus* DEX/INDO 10– day treatment protocol were compared to concentrations just prior to the start of treatment by Student's paired t–test. Statistical analysis was conducted using the SigmaStat 2.0 program (Jandel Scientific Software, San Francisco, CA), and significance was accepted at $P < 0.05$.

RESULTS

Inoculation to Delivery Interval and Gestational Length

There is evidence for a clear and uniform prolongation of *in utero* fetal survival after antimicrobial treatment, with a mean *inoculation–to–delivery* interval of 20.9 ± 1.4 days *vs.* 13.7 ± 2.5 days, respectively, ($P<0.05$), and a two to three–fold increase in the percentage of undelivered animals at 18–20 days after inoculation compared to untreated animals with IAI (Figure 1). A significant increase in the *inoculation–to–delivery* interval was noted in animals treated with AZI alone compared to untreated IAI animals (22.4 ± 2.0 *vs.* 13.7 ± 2.5 days, respectively), while there were no significant differences between AZI *plus* DEX/ INDO and IAI animals (19.4 ± 1.5 days *vs.* 13.7 ± 2.5 days, respectively). No difference was observed between the AZI and AZI *plus* DEX/INDO treated animals (22.4 ± 2.0 *vs.* 19.4 ± 1.5 days, respectively), therefore data were combined for statistical purposes and illustration in Figure 1. The average gestational age at intra–amniotic inoculation with *U. parvum* was 128 days (range: 124–135dGA) and did not differ statistically among groups; *U.parvum* alone, 130 ± 2.4 days (n=6); *U.parvum* with AZI alone, 127 ± 1 day (n=5); *U.parvum* with AZI *plus* DEX/INDO treatment, 128 ± 2 day (n=5). The mean *inoculation-to-treatment* interval was 6.2 ± 0.8 days (range: 6–8 days) and did not differ significantly between AZI alone and AZI *plus* DEX/INDO treatment groups (6.0 ± 0.8 *vs.* 6.4 ± 0.9 days, respectively). While the primary criterion for the initiation of treatment was 6–8 days of infection to provide consistency, only animals that also had a concomitant increase in uterine activity and/or change in cervical dilation (as determined by a modified Bishop's score over a 24h observational period) were included in the analysis. All animals receiving antibiotics (with or without anti–inflammatory agents) completed the 10–day regimen, except for one monkey, which completed five days of AZI *plus* DEX/INDO treatment, followed by premature rupture of membranes and cesarean section delivery at 137dGA. All treated and untreated animals delivered live fetuses. Despite prolongation of *in utero*

survival by antimicrobial therapy and an average delay in preterm birth of more than 7 days (range: 5–15 days), treated animals delivered approximately 2 weeks before term (149 ± 1.8 days) compared to instrumented historical saline or media control animals (161 ± 1.5 *or* 154 ± 2.5 days, respectively).[7]

Uterine Activity, Amniotic Fluid Cytokines, Prostaglandins and Leukocytes

Before intra–amniotic inoculation the uterus was quiescent with the average 24h HCA below 300mmHg.sec/h. Following *U. parvum* IAI there was a progressive increase in uterine contractions which reached mean peak levels of 1295 ± 228mmHg.sec/h within 6–8 days and/or prior to the initiation of antibiotic treatment (Table 1, *P*<0.05; n=16). *U. parvum* IAI also led to a significant influx of leukocytes into the amniotic fluid, and up– regulation of pro–inflammatory cytokine concentrations (TNF–α, IL–1β, IL–6, and IL–8) and prostaglandins E_2 and $PGF_{2\alpha}$ (Table 1, *P*<0.05; n=16). Both AZI alone and AZI *plus* DEX/INDO treatment significantly reduced amniotic fluid concentrations of pro–inflammatory mediators compared to pre–treatment levels (Table 1; *P*<0.05). A greater reduction was observed with AZI *plus* DEX/INDO treatment than with AZI alone, especially in regards to prostaglandin and cytokine concentrations (Table 1). However, the significant inhibitory effect on pro–inflammatory mediators (in both groups) was confined to the 10–day treatment interval. Subsequently, within several days (range: 1–12 days) after cessation of treatment, we observed a secondary rise in amniotic fluid leukocytes and pro–inflammatory mediators (data not shown), coinciding with an increase in uterine activity which culminated in preterm labor and cesarean section delivery.

Azithromycin (AZI) Concentrations

The temporal relationships of maternal plasma and amniotic fluid AZI concentrations (ng/ml) with *U. parvum* colony counts (CFU/ml) in a representative animal are illustrated in Figure 2. Data were collected after the *initial* dose of AZI demonstrate maternal plasma mean peak levels of 3239 ± 520 ng/ml were achieved within 1h, while corresponding mean peak amniotic fluid AZI concentrations reached 56.16 ± 25 ng/ml within 7h. Frequent sampling from two fetuses demonstrated peak plasma levels of 85.5 ± 9.5ng/ml within 1.2h of the *initial* maternal AZI dose, indicating a placental transfer rate of approximately 2–3%. Multiple AZI dosing (12.5mg/kg, q12h, IV for 10 days) resulted in sustained trough levels of 75–300ng/ml in the maternal circulation and amniotic compartment. At steady-state, inhibitory concentrations of AZI in the amniotic fluid equaled or exceeded maternal plasma levels at the completion of the 10–day treatment protocol and during the *washout phase*, suggesting accumulation and an extended therapeutic window within the amniotic compartment (Figure 2). A slow decay in the amniotic fluid AZI washout occurs and is likely due to extended tissue penetration. The estimated $t_{1/2}$ life after multiple dosing of AZI was 2.6 days in maternal plasma and 7.5 days in the amniotic fluid. At cesarean section delivery, fetal umbilical cord blood AZI levels were approximately 18% of maternal plasma levels (51.5 ± 20.6ng/ml *vs.* 291 ± 158.4ng/ml, respectively; n=6).

U. parvum Cultures & PCR Analysis

All animals were screened by culture and PCR for naturally occurring genital mycoplasmas *prior* to inoculation and results were all negative. Periodic amniotic fluid cultures for facultative or anaerobic bacterial infections remained negative throughout the study. None of the mothers with *U. parvum* IAI had positive blood cultures (Table 2), were febrile (with rectal temperature >102°F/38.9°C), or had systemic leukocytosis. After inoculation of *U. parvum* (7–14×10^7 CFU/ml), and initial dilution with amniotic fluid, there was an exponential growth of microorganisms which peaked at $1.91×10^6$ (CFU/ml) within 3 days (range: 1–14 days) and stabilized thereafter at $1.09×10^6$ (CFU/ml; Figure 3) prior to antibiotic treatment. Amniotic fluid *U. parvum* (CFU/ml) peaked in each animal prior to the

commencement of antibiotic treatment and were rapidly reduced by maternal AZI administration within 24h (Figure 3). Intra–amniotic *U. parvum* infection was cleared within 4 days (range: 2–7 days; n=10) after repeated maternal AZI dosing, irrespective of adjunctive anti–inflammatory treatments (Figure 3). There was no significant difference in the *inoculation–to–peak* (CFU/ml) intervals among animals or the *time–to–zero* (CFU/ml) from the start of antibiotic treatment.

In the absence of antimicrobial therapy, fetal lung, placenta, fetal membranes and amniotic fluid were 83–100% positive for *U. parvum* by culture and PCR at the time of delivery (Table 2). Fetal cord blood was positive by PCR but not culture in 1 of 6 fetuses (17%). Fetal brain and CSF remained negative by culture but CSF tested positive in 1 of 4 fetuses (25%) by PCR. The PCR results in 2 of 5 fetal brain samples from *U. parvum* infected animals were inconclusive due to unresolved assay inhibitors, and in 2 fetuses CSF samples were not obtained or were contaminated by blood. At delivery, amniotic fluid samples, placental and fetal tissues from treated animals were negative by culture in 90% of cases (Table 2, n=10). However, one animal treated with AZI *plus* DEX/INDO presented with re–growth of *U. parvum* at delivery despite clearance of microorganisms during the antibiotic treatment protocol (as determined by negative culture and PCR), suggesting the possibility of persistent organisms in biofilm.[45] Some treated animals were PCR positive but culture negative and may reflect the fact that DNA from nonviable organisms may still amplify for variable periods of time.

Histopathology of the Fetal Lung and Placental Membranes

Fetuses infected *in utero* with *U. parvum* demonstrate a progressive alveolitis and bronchiolitis (Figure 4). An exudative pneumonia develops within days after infection characterized by a diffuse presence of neutrophils and macrophages in the alveolar spaces, a stimulation of Type II pneumocytes and areas of necrosis in alveolar and terminal airway epithelium. In more prolonged ureaplasmal infection (>10 days) the acute inflammatory response is followed by lymphocytic infiltration of alveolar septae, prominent hyperplasia and hypertrophy of Type II pneumocytes, reactive proliferation of epithelial cells and an apparent thickening of alveolar walls (Figure 4B). In longer-term infection (>15 days) there is also the conspicuous development of peribronchiolar lymphoid tissue aggregates accompanied by hyperplasia and hypertrophy of bronchiolar epithelium (Figure 4C). Maternal AZI treatment initiated after 6–8 days of *U. parvum* IAI (irrespective of anti–inflammatory treatments) substantially reduced the magnitude and distribution of intra–alveolar leukocytes, *albeit* scattered foci of amniotic debris and clumps of leukocytes remained (Figure 4D). There was also a post–treatment reduction in alveolar wall leukocytic infiltration, in Type II pneumocyte hyperplasia and in the overall appearance of alveolar wall thickness (Table 4). It was notable that maternal AZI treatment prevented the development of peribronchiolar lymphocytic aggregates which were absent or rare in AZI treated animals, and if present were much smaller and not accompanied by mucosal hyperplasia of the bronchiolar epithelium.

Histologic funisitis was not commonly observed in our cohort, however one of six animals (17%) in the untreated *U.parvum* IAI group clearly demonstrated funisitis and mild chorionic vasculitis following 21 days of *U. parvum* IAI (Figure 5). In general, we observed the degree of the fetal inflammatory response (i.e., histologic chorionitis, chorioamnionitis, funisitis) was dependent on the duration of intra–amniotic exposure to *U. parvum*, but there were too few cases within each diagnostic category for statistical analysis.

As we have previously described *U. parvum* IAI results in an acute chorioamnionitis followed by a subacute inflammatory response in the extra–placental fetal membranes and in the superficial parietal decidua.[7] Similarly, the amnion/chorion demonstrated edematous

thickening and marked infiltration of polymorphonuclear leukocytes throughout the connective tissue layers and in the chorion trophoblast layer following *U. parvum* IAI (Figure 5). Chorion trophoblast cells typically showed signs of necrosis and microabscess formation. The predominant effect of maternal antimicrobial therapy was to reduce the extent and severity chorioamnionitis. Histologic evidence of IAI was absent in 4 of 10 treated animals. Although histologic evidence of IAI was still present in AZI alone or AZI *plus* DEX/INDO treatment in 6 of 10 animals, the degree of inflammation was markedly less with more improvement in AZI *plus* DEX/INDO treated animals compared to AZI alone (4 of 5 *vs.* 2 of 5, respectively, Figure 5).

COMMENT

We have previously demonstrated that intra–amniotic inoculation of *U. parvum* (10^7 CFU/ml, *serovar I*) increases uterine contractility, pro–inflammatory cytokines and prostaglandins E_2 and $F_{2\alpha}$ and leads to preterm delivery in a nonhuman primate model.[7] These observations are consistent with previously reported human data.[15–17, 20–23] Histopathologic findings of chorioamnionitis, a systemic fetal inflammatory response and pneumonitis worsen with the duration of *in utero* ureaplasma infection.[7] Our current study is the first to show that antenatal AZI, multi–dose IV treatment protocol is effective in clearing *U. parvum* IAI by virtue of an extended therapeutic window and a prolonged elimination phase in the amniotic fluid. *U. parvum* colony counts (CFU/ml) were reduced by 90% within 24 hours of maternal AZI dosing and viable bacteria were effectively eliminated from the amniotic fluid on average in 4 days. In contrast to our previous work with group *B streptococcus* (GBS) IAI,[35] we found that adjunctive anti–inflammatory treatment for *U. parvum* IAI had no effect in prolonging the latency period (*inoculation–to–delivery*) compared to antibiotic treatment alone. These differing results are probably explained by the inherent anti–inflammatory properties of azalide antibiotics which accumulate preferentially in leukocytes and macrophages[46] and inhibit production of pro–inflammatory cytokines.[47–49]

Antenatal indomethacin (INDO) was the first non-steroidal anti-inflammatory drug used clinically for tocolysis. Our decision to utilize INDO in our chorioamnionitis model as opposed to selective COX-2 inhibitors is based on the following: (1) a large body of experimental data and our familiarity with INDO in rhesus monkeys; (2) evidence that selective COX-2 inhibitors have fetal renal or cardiovascular side effects; (3) evidence that the anti-inflammatory actions of INDO involve mechanisms other than COX-2 inhibition (e.g., cerebral production of prostaglandins, which is dependent on COX-1 may not be suppressed by selective COX-2 inhibitors. In addition, INDO has antioxidant and hydroxyl radical-scavenging properties, functioning as a peroxisome proliferator-activated receptor-gamma (PPARγ) agonist. PPARγ agonists have been shown to reduce the expression of cytokines and neuronal damage in cell culture and animal models[50]. Clinical experience demonstrates that limiting the duration (five-day regimen) and discontinuing INDO before delivery is anticipated, reduces the frequency of serious adverse fetal side-effects (i.e, oligohydramnios, impaired renal function, premature closure of the ductus arteriosus). Although prostaglandin blockade is an effective therapeutic approach, a more comprehensive strategy may be to also inhibit the pro-inflammatory cascade upstream. Clinical examples include bacterial meningitis in which dexamethasone (DEX) adjuvant therapy reduced late sequelae such as deafness or Crohn's disease. Furthermore, DEX exerts a protective effect on potential side-effects of INDO (e.g., intraventricular hemorrhage) and on cytokine-induced damage of oligodendrocyte precursor cells[51]. Since INDO and DEX readily cross the placenta, target multiple pro-inflammatory effectors and are currently used perinatal agents, they represent suitable candidates to use in the multi-factorial management of infection-associated preterm labor. A recent report suggests long-term neurologic

outcome may actually be improved if the use of INDO is accompanied by antenatal steroids such as DEX.[52]

In the current study, despite prolongation of gestation by antibiotic treatment (with or without anti–inflammatory agents), neither *U. parvum* nor GBS treated[35] animals achieved term pregnancy, suggesting that antibiotics have limited efficacy in resolving chorioamnionitis, and as a consequence a pro–inflammatory signal for preterm delivery persists in the fetal membranes and/or maternal decidua. Indeed, two antibiotic trials using combinations of clindamycin, erythromycin, metronidazole, and ampicillin to prevent preterm delivery, reported no difference in histological chorioamnionitis among antibiotic treatments *vs.* placebo.[53, 54] We found that AZI alone or combined with anti–inflammatory treatments were associated with an observable reduction in the frequency and degree of histologic chorioamnionitis. However, in many cases residual chorioamnionitis persisted and may be related to the resumption or recurrence of preterm labor. Preliminary evidence based upon fluorescence *in situ* hybridization (FISH) to identify the fetal or maternal origin of leukocytes, suggests both maternal and fetal cells enter the amniotic fluid during IAI (data not shown), but the increase in fetal leukocytes is proportionately greater ($p<0.05$). During AZI treatment total leukocyte numbers and the fetal contribution declined substantially, suggesting attenuation of the *fetal* immune response during *maternal* antibiotic therapy and provides further evidence for *in utero* treatment of the fetus. The rebound in amniotic fluid leukocytes, pro–inflammatory mediators and uterine activity (which occurred 4–10 days after completion of therapy) is driven by maternal host–defense immune mechanisms.[55] Relevant clinical observations have been reported by Hassan *et al.*[56] in four patients with a sonographic short cervix and positive amniotic fluid cultures for *Ureaplasma* spp. All were treated with maternal AZI (IV, for 7 days). Follow–up cultures were negative at repeat amniocentesis, with three patients delivering at or near term (two without histologic chorioamnionitis) but the fourth patient delivered preterm with negative cultures but elevated numbers of amniotic fluid leukocytes and histologic evidence of chorioamnionitis.[56]

Our experimental results and the clinical reports noted above support the hypothesis that a persistent and widespread inflammatory response in the chorion/decidua is responsible for triggering the post treatment recurrence of preterm labor, given the absence of viable bacteria in the amniotic fluid or fetal membranes at delivery. A choriodecidual inflammatory mechanism is consistent with recent experimental evidence in nonhuman primates that choriodeciduitis represents a transitional stage of intrauterine infection which may be self–limited, remain dormant or progress to IAI. Coupled with clinical observations, these data suggest that choriodecidual inflammation is a precursor of the preterm labor syndrome.[34] Although at its inception, choriodecidual inflammation is typically localized to a discrete site in the lower pole of the uterus, Kim *et al.*[57] have shown in human studies that microbial invasion of the amniotic cavity is followed by diffuse and widespread inflammation in chorion/decidua.[57] It is interesting that the maternal antimicrobial and anti–inflammatory therapy as administered in our protocol was more effective in resolving the large amniotic and fetal bacterial load (and in reducing the fetal inflammatory response) yet was found lacking in the resolution of choriodecidual inflammation. It is also apparent from our results that immunomodulators given in tandem with antibiotics may not always act synergistically in the management of preterm labor associated with IAI, depending upon the nature of the microbial pathogen and the anti–inflammatory characteristics of a given antibiotic. In future investigations it will be necessary to develop newer modalitiesof anti–inflammatory agents or to administer them sequentially with antimicrobials (or in repeated doses) in order to preempt the consequences of residual inflammation in the fetal membranes and decidua. Newer classes of immunomodulators, such as specific cytokine inhibitors or TLR receptor antagonists, may yet confer additional benefits to women in preterm labor or potentially

ameliorate fetal lung and brain injury.[58] Answers to these critical questions will require further detailed study.

Bronchopulmonary dysplasia (BPD) is an important consequence of prematurity and can lead to the development of chronic lung disease.[7, 13, 14, 59] Human infants colonized with *Ureaplasma* spp. at birth subsequently have a 43% incidence of developing BPD in contrast to infants without *Ureaplasma* spp. colonization, that have an incidence of approximately 19%.[60, 61] *Ureaplasmal* infection of the respiratory tract promotes a pro–inflammatory cytokine cascade and can also block the expression of the regulatory cytokines, IL–6 and/ or IL–10.[62] A meta–analysis of 23 cohort studies (published between 1988 and 2004) demonstrated a significant association between *Ureaplasma* spp. infection and subsequent development of BPD at 28 days ($P < 0.001$) and 36 weeks ($P < 0.001$).[63] *Ureaplasma* infection initiated *in utero* and augmented by postnatal exposure to volutrauma and oxygen elicits a sustained, dysregulated inflammatory response in the immature lung that impairs alveolarization, and stimulates myofibroblast proliferation and excessive collagen and elastin deposition.[13] Reproduction of histologic lesions in lungs of newborn mice, baboons and rhesus macaques by *Ureaplasma* spp. provides compelling evidence that this organism is a cause of neonatal bronchiolitis and pneumonia.[7, 14, 64] Intranasal or intratracheal inoculation with *Ureaplasma* spp. causes an acute bronchiolitis in preterm neonatal baboons, with similar findings being observed in a premature baboon model exposed to brief antenatal colonization followed by postnatal ventilation.[65]

We have previously demonstrated that rhesus monkey fetuses infected *in utero* with *U. parvum* demonstrate a progressive alveolitis and broncholitis which is related to the duration of IAI.[7] Similarly, in our current study, prolonged *ureaplasmal* IAI (>10 days) was characterized by reactive proliferation of type II pneumocytes, lymphocytic inflitration of alveolar walls and conspicuous peribronchiolar lymphocytic aggregates accompanied by hyperplasia of the overlying epithelium in terminal airways (Figure 4). It is anticipated histopathologic changes (if present after birth) would produce altered lung compliance and impairment in the functional properties of the neonatal lung. It is noteworthy that similar lesions were absent or substantially reduced in fetal lungs when maternal AZI therapy (with or without DEX/INDO) was initiated 6–8 days after the onset of *U. parvum* IAI. There were no major differences in the histopathologic scoring of fetal lung lesions with the addition of DEX (Table 3), which may suggest only a marginal effect of DEX treatment in our study protocol. However, it is possible that the actions of DEX are masked by AZI treatment due to the inherent anti–inflammatory properties of this class of macrolide antibiotic, thus making it difficult to tease out the specific impact of DEX treatment in the context of lung maturation. In order to overcome these limitations in our treatment protocol it will may be necessary to administer anti–inflammatory agents sequentially with antimicrobials (or in repeated doses) to fully appreciate the beneficial role anti–inflammatory co–treatments might serve in the management of preterm labor associated with IAI. It is evident that the antibiotic regimen employed was effective in resolving acute and subacute inflammatory changes in the fetal lung, and in preventing the development of more advanced respiratory tract lesions (e.g., peribronchiolar lymphocytic aggregates and associated epithelial hyperplasia). To what extent these improvements in histopathologic sequelae of *Ureaplasma* IAI will translate into improved pulmonary function in the neonate remains to be determined; this is the focus of future studies being carried out by our research group.

The high rate of mortality and respiratory morbidity associated with *Ureaplasma* colonization of preterm infants highlights the need to initiate effective treatment strategies as soon as possible after birth or preferably prenatally. However, trials of erythromycin in *Ureaplasma* colonized preterm infants have not demonstrated efficacy to prevent BPD or to eradicate respiratory tract colonization.[13] Azalide antibiotics such as AZI exhibit higher

potency than erythromycin against *Ureaplasma* spp. isolates *in vitro* and are concentrated in alveolar fluid and macrophages. AZI, but not erythromycin prophylaxis, reduced inflammation and improved outcomes in a murine neonatal *Ureaplasma* infection model.[66] A phase I neonatal clinical PK and safety trial showed that there were no serious adverse events attributed to AZI (10mg/kg for 3 days), *albeit* insufficient to eradicate *Ureaplasma* spp. (43% failure rate to prevent BPD).[67] In addition, neonatal AZI administration did not suppress inflammatory cytokines or myeloperoxidase activity in tracheal aspirates.[67] However, a dose of 20mg/kg/day for 3 days (which approximates the regimen we report for antenatal therapy) was predicted to maintain neonatal AZI plasma concentrations above the MIC_{50} for *Ureaplasma* spp.[67] Because the lung injury mediated *in utero* by *Ureaplasma* is potentially augmented by postnatal mechanical ventilation and hyperoxia, we speculate that antimicrobial therapy initiated during pregnancy would be optimal.

AZI is structurally related to erythromycin, but has prolonged duration of action, improved tissue penetration and an extended range of antimicrobial coverage against *Ureaplasma* spp.[68–70] There is uncertainty regarding the therapeutic efficacy of AZI in women due to limited placental permeability and the lack of pharmacodynamic data in human pregnancy.[36, 71] Studies in pregnant women have been limited to oral dosing of AZI given to multiple subjects at varying intervals prior to delivery.[36] Our study is the first to incorporate a multi–dose regimen for a period of 10 days accompanied by intensive PK sampling of both maternal plasma and amniotic fluid in pregnant nonhuman primates with experimental *U. parvum* IAI. The maximum AZI concentrations determined for maternal plasma in this study was approximately 10 times higher than concentrations attained after a standard oral dose of 500–1000mg in pregnant women (4.1mg/ml *vs.* 0.31–44mg/ml, respectively) (Pfizer. Zithromax Package insert, 2009).[36] Intensive sampling during the *initial* AZI dose followed by PK modeling demonstrated that the rate of AZI transfer from the maternal circulation to the intra–uterine compartment was approximately 2–3% of the rate of penetration into peripheral tissues,[39] and is comparable to previously published human data.[36, 72] Within the first 24h of maternal AZI treatment the fetal exposure levels (AUC, area under the PK curve) were 6.2% of the maternal exposure; with repeated maternal dosing, fetal exposure increased to 14.3% of maternal exposure levels.[39] Although there was a large difference between AZI mean peak levels in maternal plasma and amniotic fluid, steady–state trough concentrations were similar or higher in the amniotic fluid suggesting accumulation (Figure 2). Two-compartmental PK analysis performed in tandem with this data set has provided an estimate of the rate of transfer between the maternal circulation and the amniotic compartment; the steady accumulation of AZI in the amniotic fluid compartment during multiple dosing was attributable to the rate of drug entry in to the amniotic compartment which exceeded the rate of clearance by more than two-fold.[39] Furthermore, a prolonged elimination phase of AZI in the amniotic fluid (terminal $t_{1/2}$ of 7.5 days) contributes to an extended therapeutic window. The concurrent PK modeling also provided the concentration of AZI required to eradicate 50% of *U. parvum* from the amniotic fluid (EC_{50}); an accurate assessment is normally confounded by the growth and pH conditions required *in vitro*. Using a sigmoid inhibitory model, the EC_{50} was determined to be 29ng/ml.[39] Therefore, at steady–state, a maternal dose of AZI (12.5mg/kg, q12h, IV for 10 days) would yield a maximum concentration of 160ng/ml in the amniotic fluid which would be in excess of the EC_{50} by 5.3-fold.[39] Moreover, AZI is concentrated 10 to100–fold intracellularly, particularly in leukocytes and macrophages,[36] thus effective inhibitory levels of AZI are likely to be achieved within tissues. Although we have not yet confirmed AZI concentrations in rhesus fetal tissues, they are expected to exceed effective inhibitory levels for AZI by analogy with tissue data from women after oral dosing[36] and by the favorable clearance of *U.parvum* from placenta and other fetal tissues reported here (Table 2).

Our data support the proposition that *antenatal* AZI therapy can eradicate *U. parvum* from the amniotic fluid and key fetal organs, with subsequent prolongation of pregnancy which provides a window of opportunity to effectively reduce the severity of lung injury *in utero*. These interventions may subsequently reduce the incidence of postnatal infections and attenuate the neonatal systemic inflammatory response with a significant impact on the overall rate of adverse outcomes and/or disability among premature survivors. In contrast to fetal and neonatal lung injury, the linkage between exposure to *Ureaplasma* spp. and neurologic sequelae is less understood. Cases of intraventricular hemorrhage (IVH) have been linked to *Ureaplasma*–positive CSF. In a recent study the risk of severe IVH was 5–fold higher in serum PCR–positive than PCR–negative infants after adjustment for gestational age.[11] A role for *Ureaplasma* spp. in the development of cerebral white matter injury is supported by recent findings in a murine intra–uterine infection model in which the brains of fetal and newborn mice showed evidence of microglial activation, delayed myelination and disturbed neuronal development.[73] We previously reported no gross or microscopic evidence of hemorrhage or leukomalacia in brains of immature rhesus macaque fetuses with *U. parvum* positive cultures from CSF or brain tissue.[7] However, subsequent studies using specific immunofluorescence techniques demonstrated that prolonged *U. parvum* IAI exposure (14–21 days) is associated with areas of moderate to severe microgliosis and astrocytosis and scattered foci of neuronal injury in the periventricular white matter region.[74] There is growing concern that prolongation of *in utero* existence in the presence of infection/inflammation is potentially deleterious to the fetal brain. Although the primary objective was to assess fetal lung injury, our preliminary observations suggests that early maternal AZI therapy alone or combined with anti-inflammatory agents may also reduce or prevent the development of brain inflammation and neuronal injury.[74] Additional studies are needed to define the contribution of intra–uterine *Ureaplasma* IAI on cerebral white matter injury and neurodevelopmental outcome; such studies are currently underway in our nonhuman primate model.[75, 76]

Our findings hold promise for the development of appropriate treatment strategies which may alter the course of preterm labor as a consequence of ascending infection. The eradication of *Ureaplasma* spp. from fetal tissues by antenatal antibiotic therapy suggests the feasibility of reducing fetal lung injury, and by extension reducing the risk of adverse neonatal outcome and subsequent development of BPD/chronic lung disease. Future investigations to prevent prematurity should be directed toward identification and localization of specific microorganisms, combined with targeted antibiotic trials to determine whether such interventions can improve long–term perinatal outcomes.

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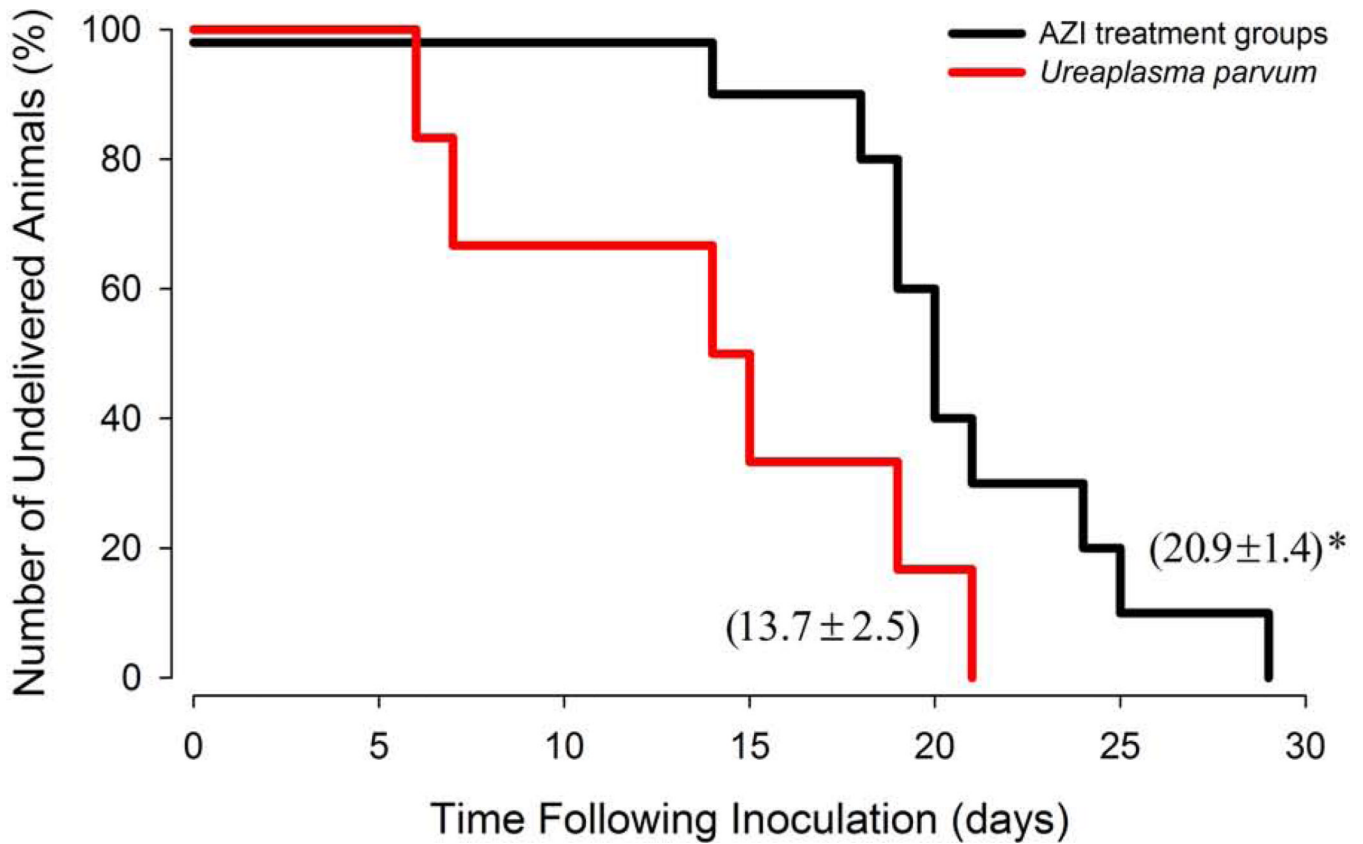
Figure 1. Kaplan–Meier Plot Illustrating the Percentage of Animals Remaining Undelivered in Days Following Intra–Amniotic Inoculation of *U. parvum*

The survival analysis compared data for *U. parvum* untreated animals (red line, n=6) with the combined data for AZI and AZI *plus* DEX/INDO treated animals (black line, n=10). There is a clear and uniform prolongation of *in utero* fetal survival and an increase in the mean *inoculation–to–delivery* interval in the AZI treated animals compared to untreated *U. parvum* IAI (20.9 ± 1.4 days *vs.* 13.7 ± 2.5 days, respectively; *P*<0.05).
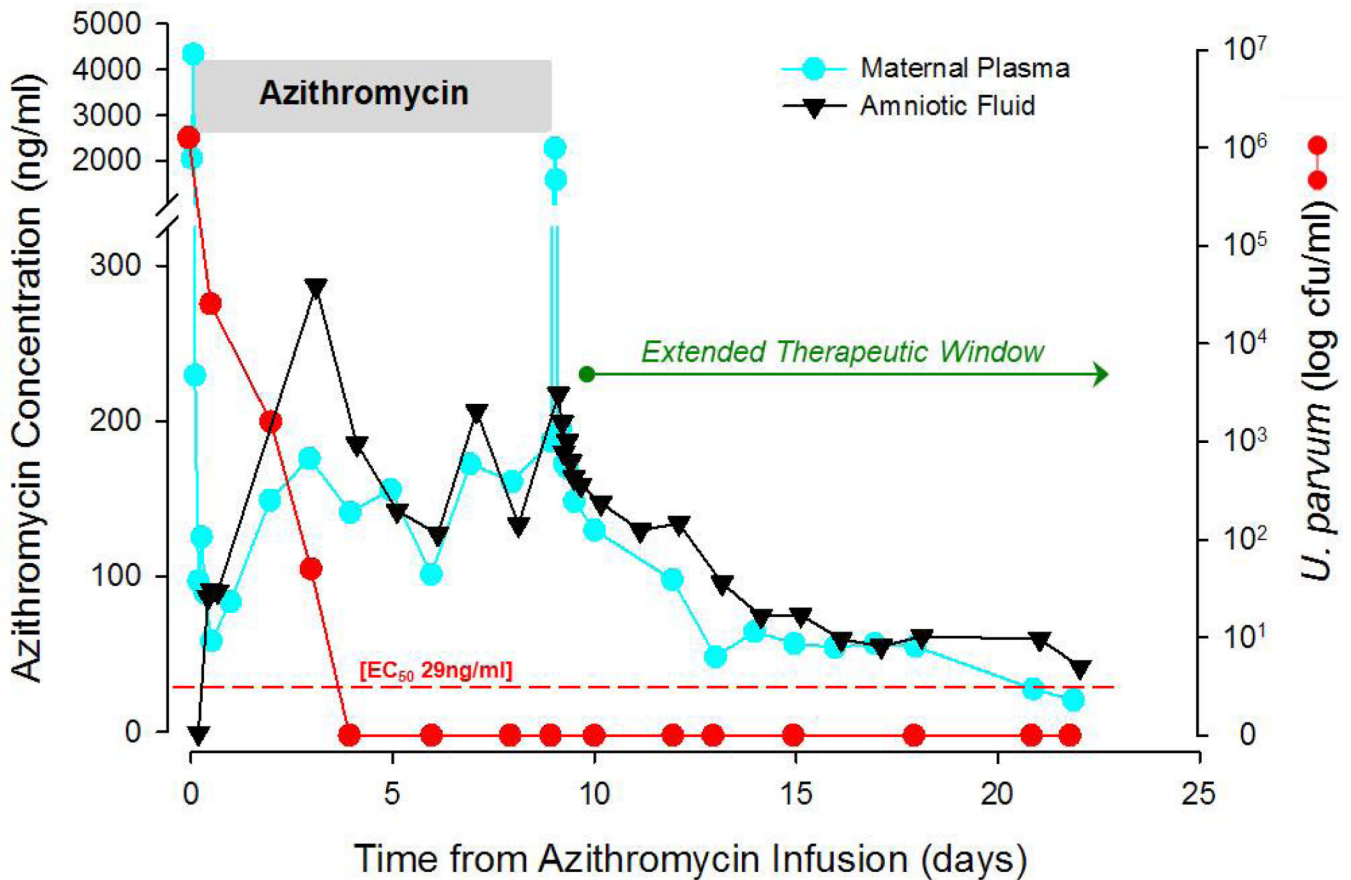
Figure 2. Temporal Relationship of Maternal Plasma and Amniotic Fluid Azithromycin (AZI) Concentrations

A representative animal illustrating maternal plasma (blue circles) and amniotic fluid (black triangles) AZI concentrations (ng/ml) during maternal multi–dosing (12.5mg/kg, q12h, IV for 10 days) and subsequent washout period. *Ureaplasma* colony counts (CFU/ml) are also depicted (red circles). Frequent sampling was performed at the beginning and end of AZI administration; daily samples were obtained during the intervening days of AZI infusion (gray shaded bar) and in the A.M. after cessation of treatment (AZI *washout* period). Steady–state levels of AZI (75–300ng/ml) in the amniotic fluid equaled or surpassed maternal plasma concentrations at the end of the 10–day treatment interval, thus indicating accumulation of AZI in the amniotic fluid which provides an extended therapeutic window. At stead–state, AZI inhibitory concentrations in the amniotic fluid exceeded the EC_{50}, the concentration needed to eradicate 50% of *U. parvum*, by 5–fold (red dashed line).
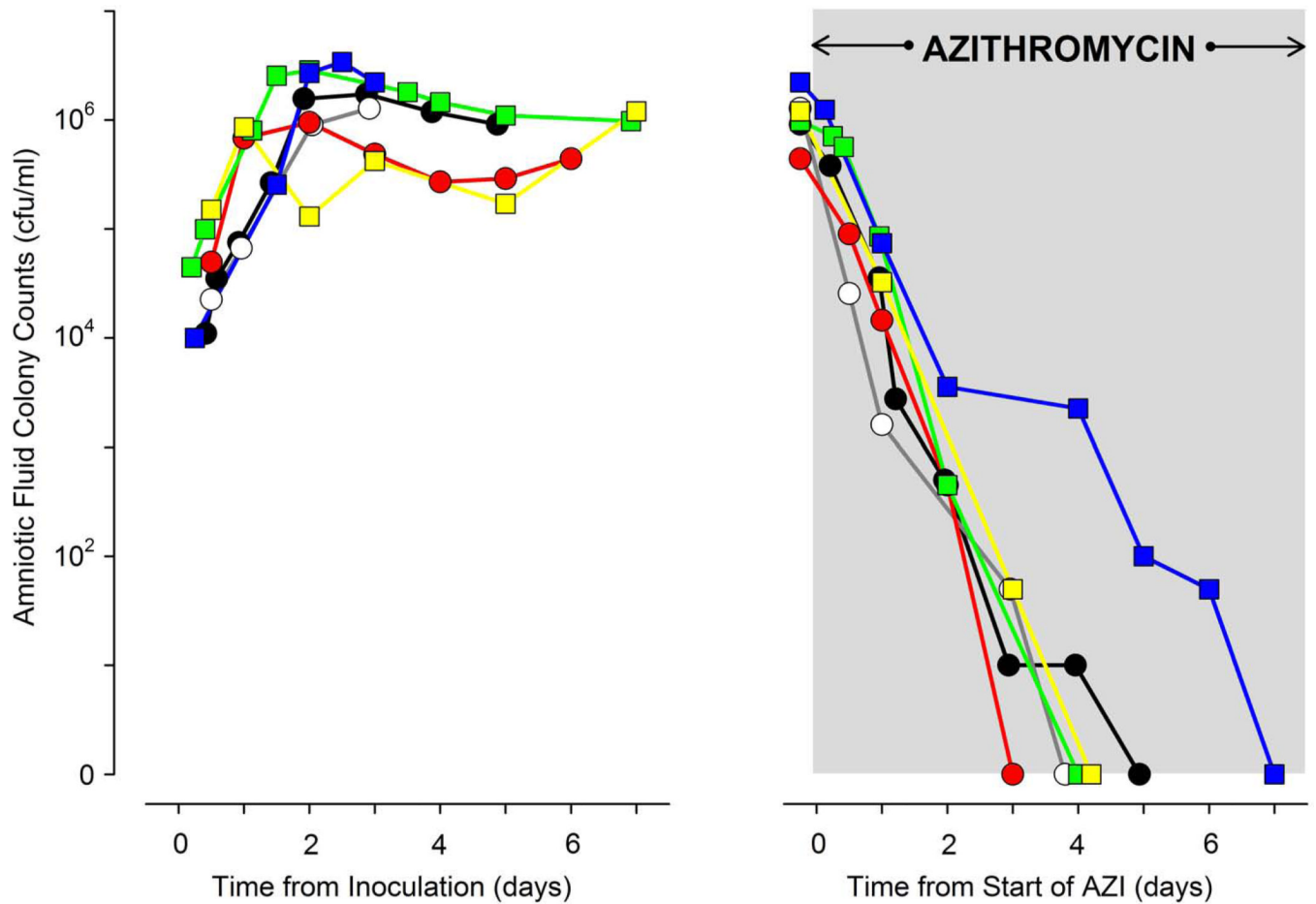
Figure 3. Amniotic fluid *U. parvum* (CFU/ml) Growth Curves (log scale)
A representative group of animals from each AZI treatment group is shown (AZI alone, *colored circles*, n=3 and AZI *plus* DEX/INDO, *colored squares*; n=3) from *inoculation–to–treatment* and from the start of *treatment–to–clearance* (gray shaded area). In all animals, amniotic fluid CFU/ml peaked before the start of treatment and declined rapidly in the first 24h of AZI treatment. *U. parvum* microorganisms were eradicated from the amniotic fluid within 4 days (range 2–7 days).
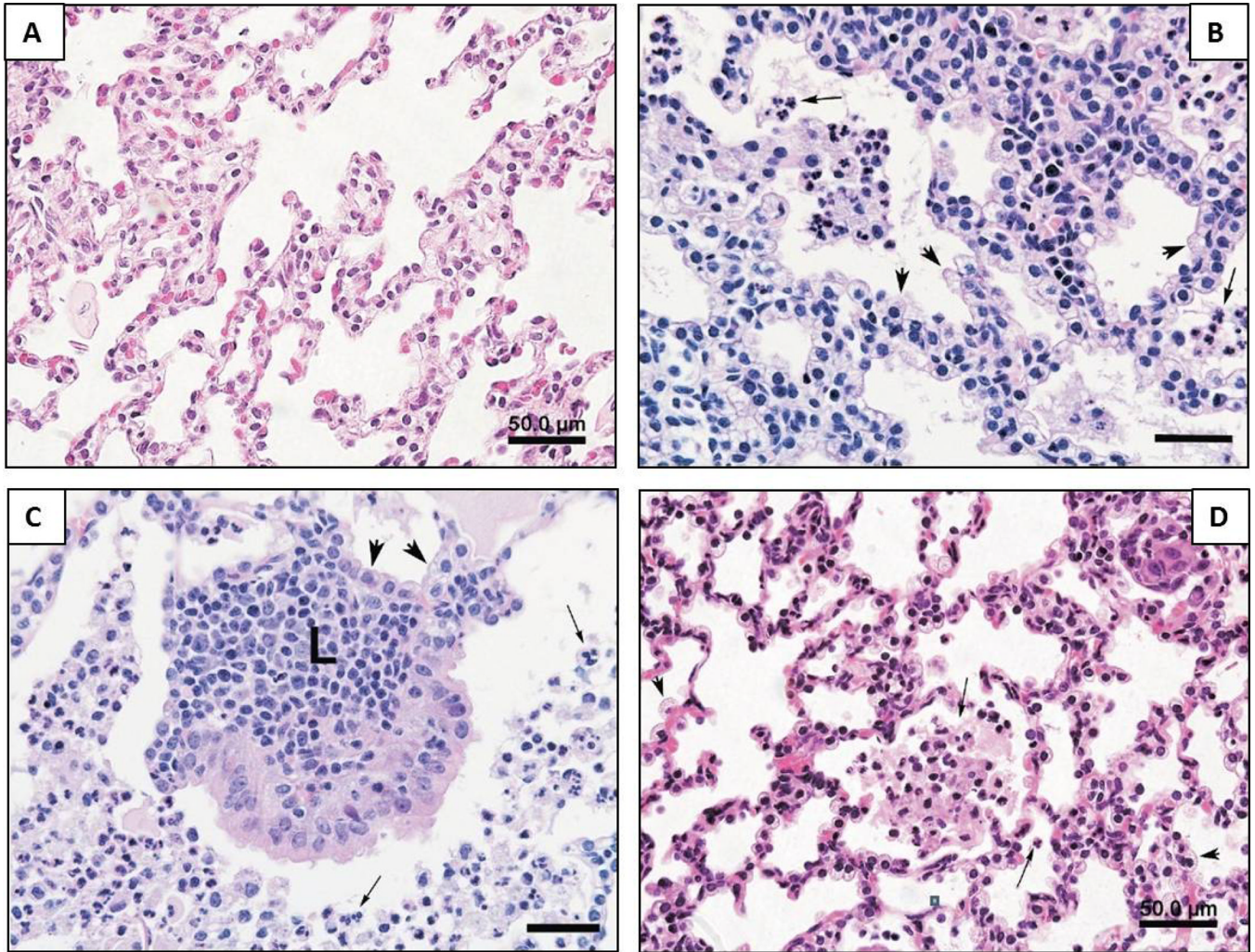
Figure 4. Histopathology of the Fetal Lung

Comparative evaluation of fetal lung histology among *U. parvum* IAI and antimicrobial treatment animals is illustrated. **(A)** Control non–infected lung at 131dGA showing partial atelectasis and absence of alveolar inflammatory cells; **(B)** *U. parvum* IAI for 15days (153dGA) demonstrating hyperplasia and hypertrophy of type II pneumocytes (arrow head); **(C)** *U. parvum* IAI for 21days (146dGA) illustrates large peribronchiolar lymphocytic aggregates and hyperplasia of bronchiolar epithelium (L); **(D)** *U. parvum* IAI for 7days (155dGA) treated with AZI *plus* DEX/INDO showing resolution of pneumonitis with residual leukocytes in respiratory bronchioles and alveoli (arrows) and an absence of lymphocytic aggregates; (H&E slides; mag. 400×, scale bar = 50μm).
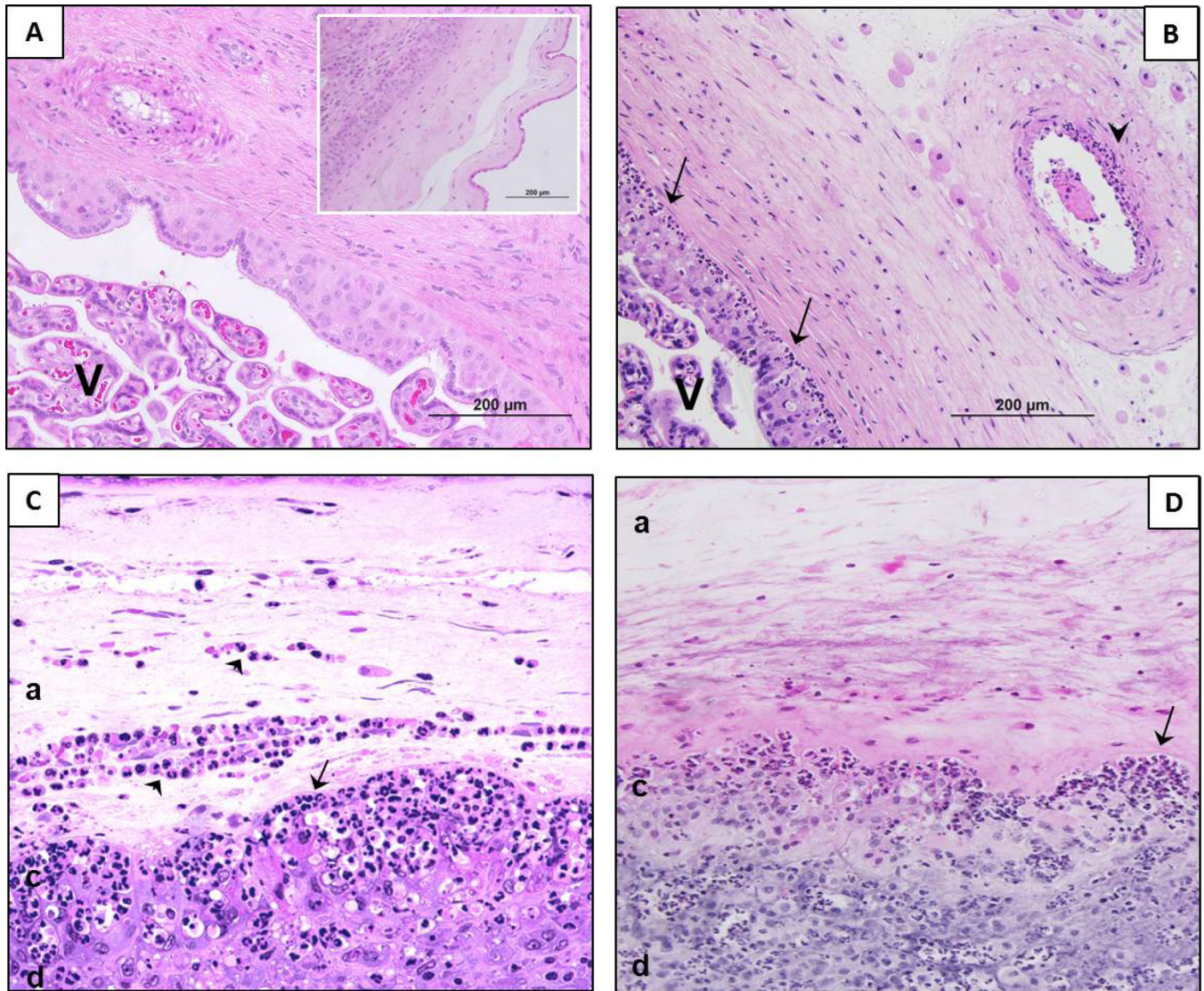
Figure 5. Histopathology of the Placenta and Fetal Membranes
Microscopic appearance of the chorionic plate **(A)** and fetal membranes (*insert*) from a saline control animal near term demonstrating intact amnion epithelium and collagen layer with absence of leukocytic infiltration; **(B)** Following 21 days of IAI, accumulation of neutrophils was observed marginating into the chorion (arrows), which represented the minimal criteria for histologic evidence of IAI. There was also a fetal vascular inflammatory response (arrow head); **(C–D)** Marked histologic evidence of infection was represented by both chorionic and amnionic layer involvement by neutrophils (arrows), defined as chorioamnionitis. The predominant effect of maternal antimicrobial therapy was to reduce the frequency and degree of the leukocytic inflammatory response in the amnionic layer **(B *vs.* C)**. (H&E slides; mag. 400×, scale bar = 200μm; Amnion = a; Chorion = c, Decidua = d).

Table 1

Mean 24hr Uterine Activity (UA) and Amniotic Fluid Prostaglandins, Cytokines and Leukocytes before and after *U. parvum* Inoculation and with AZI alone or AZI *plus* DEX/INDO Treatment.

Variable	*Ureaplasma parvum* (n=16)		AZI alone (n=5)		AZI *plus* DEX/INDO (n=5)	
	Baseline	Peak Concentration	Pre–Treatment Concentration	Post–Treatment Concentration	Pre–Treatment Concentration	Post–Treatment Concentration
UA (mmHg.sec/h)	268 ± 31	$1,295 \pm 228$[#]	969 ± 423	347 ± 120[*]	$1,451 \pm 438$	285 ± 47[*]
PGE$_2$ (pg/ml)	83 ± 13	$5,830 \pm 1,800$[#]	$2,821 \pm 1,458$	$1,145 \pm 535$[*]	$3,810 \pm 1,541$	191 ± 135[*]
PGF$_{2\alpha}$ (pg/ml)	84 ± 12	$2,099 \pm 480$[#]	$1,372 \pm 368$	556 ± 212[*]	$2,358 \pm 1,213$	166 ± 42[*]
TNF–α (pg/ml)	31 ± 20	$1,255 \pm 374$[#]	$1,227 \pm 441$	39 ± 26[*]	927 ± 155	22 ± 13[*]
IL–1β (pg/ml)	30 ± 16	859 ± 243[#]	209 ± 98	25 ± 16[*]	786 ± 251	6 ± 5[*]
IL–6 (ng/ml)	5 ± 2	98 ± 27[#]	146 ± 54	19 ± 10[*]	167 ± 34	9 ± 2[*]
IL–8 (ng/ml)	1 ± 1	56 ± 20[#]	81 ± 52	30 ± 24[*]	97 ± 22	14 ± 5[*]
Leukocytes (cells/µl)	25 ± 6	$3,015 \pm 601$[#]	$1,840 \pm 675$	594 ± 248[*]	$2,796 \pm 571$	540 ± 129[*]

Data are Mean ± SEM. Concentrations of amniotic fluid pro–inflammatory mediators (n=16) represent peak levels during IAI (range: 6–21 days), or levels prior to treatment (range: 6–8 days of IAI). Post-treatment levels represent minimum concentrations during the 10–day AZI treatment protocol.

[#] Significant compared to baseline pre–infection (*P*<0.05, n=16)

[*] Significant compared to pre–treatment (*P*<0.05, n=5 each AZI group).

Abbreviations: AZI, *Azithromycin*; DEX, *Dexamethasone*; INDO, *Indomethacin*; UA, *Uterine Activity*; PGE$_2$, *Prostaglandin E$_2$*; PGF2α, *Prostaglandin F2α*; TNF–α, *Tumor Necrosis Factor*; IL, *Interleukin*.

Table 2

Culture and PCR results for Amniotic Fluid, Maternal and Fetal Blood and Tissues obtained after *U. parvum* infection, AZI alone or AZI *plus* DEX/INDO Treatment at Cesarean Section Delivery.

Fluids and Tissues	*Ureaplasma parvum* (n=6)		Combined AZI Treatments Groups (n=10)	
	Culture (%)	PCR (%)	Culture (%)	PCR (%)
Amniotic Fluid	6/6 (100)	6/6 (100)	1/10[†] (10)	7/10 (70)
Maternal Blood	0/6 (0)	1/6 (17)	0/10 (0)	0/10 (0)
Fetal Blood	0/6 (0)	1/6 (17)	0/10 (0)	1/10 (10)
Fetal Membranes	6/6 (100)	6/6 (100)	1/10 (10)	4/10 (40)
Placenta	6/6 (100)	6/6 (100)	1/10 (10)	2/10 (20)
Fetal Lung	5/6 (83)	6/6 (100)	1/10 (10)	5/10 (50)
Fetal Brain	0/5 (0)	0/3 (0)[b]	0/10 (0)	1/10 (10)
Cerebral Spinal Fluid	0/4 (0) [a]	1/4 (25)[b]	0/9 (0)	1/9 (11)

Positive results are expressed as ratios and percentages.

[a] Fetal CSF cultures were not uniformly obtained due to contamination with blood,

[b] PCR results were inconclusive in 2 of 5 brain samples.

[†] One AZI *plus* DEX/INDO treated animal experienced re-growth of *U. parvum* at delivery despite clearance of amniotic fluid by culture and PCR during the 10–treatment interval.

Abbreviations: AZI, *Azithromycin*; DEX, *Dexamethasone*; INDO, *Indomethacin*; PCR, *polymerase chain reaction*.

Table 3

Histopathologic Scoring of Fetal Lung Tissue obtained at Cesarean Section Delivery after *U. parvum* Infection, AZI alone or AZI *plus* DEX/INDO Treatment.

	Alveolar Leukocytic Infiltration	Type II Pneumocyte Hyperplasia	Peribronchiolar lymphocytic Aggregates
Control (n=7)	1 (0–1)	0 (0–1)	0 (0–1)
Ureaplasma parvum **(n=6)**	3 (2–3)	3 (2–3)	3 (2–3)
U. parvum **plus AZI (n=5)**	1 (0–1)	1 (1–3)	1 (0–2)
U. parvum **plus AZI and DEX/INDO (n=5)**	2 (0–3)	2 (0–2)	1 (0–2)

Fetal lung tissue was scored according to a modified method by Viscardi *et al.*[25]

Abbreviations: AZI, *Azithromycin*; DEX, *Dexamethasone*; INDO, *Indomethacin*.