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Abstract

## Premature delivery in ascending placentitis is associated with increased expression of placental cytokines and allantoic fluid prostaglandins E<sub>2</sub> and F<sub>2α</sub>

M.M. LeBlanc<sup>a,\*</sup>, S. Giguere<sup>a</sup>, K. Brauer<sup>a</sup>, D.L. Paccamonti<sup>b</sup>,  
D.W. Horohov<sup>b</sup>, G.D. Lester<sup>a</sup>, L.J. O' Donnell<sup>a</sup>,  
B.R. Sheerin<sup>a</sup>, L. Pablo<sup>a</sup>, D.H. Rodgerson<sup>a</sup>

<sup>a</sup>University of Florida, Gainesville, FL, USA

<sup>b</sup>Louisiana State University, Baton Rouge, LA, USA

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### 1. Introduction

Ascending placentitis results in the premature delivery of a dead, sick or precociously mature, viable foal. The mechanism through which ascending placentitis causes premature delivery is not known. We hypothesized that premature delivery in ascending placentitis is a consequence of increased expression of pro-inflammatory cytokines in allantoic fluid and placental tissues.

### 2. Materials and methods

Two experiments were conducted to investigate this hypothesis. In Experiment 1, TNF $\alpha$ , IL-1 and IL-6 and the prostaglandins F<sub>2 $\alpha$</sub>  and E<sub>2</sub> were measured in allantoic fluid of eight mares with ascending placentitis and in four that carried normal pregnancies. Allantoic fluid was collected by allantocentesis in control mares and in four with experimental placentitis and through allantoic fluid catheters in four experimental mares that were instrumented with intra-uterine catheters 10–16 days before bacterial inoculation. Allantoic fluid was collected every 7–10 days from Day 280 of gestation (dGa) to parturition in control mares. In experimental mares, it was collected once before bacterial inoculation and then every 2–4 days after inoculation in mares with allantoic fluid catheters and every

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\* Corresponding author. Present address: Rood and Riddle Equine Hospital, 2150 Georgetown Road, P.O. Box 12070, 40580 Lexington, KY, USA.

E-mail address: Mleblanc@roodandriddle.com (M.M. LeBlanc).

4–6 days in non-instrumented mares. Ascending placentitis was induced in instrumented mares between dGa 283 and 291 by intra-cervical inoculation with  $10^8$  CFU of *Streptococcus equi* subspecies *zooepidemicus*. Non-instrumented treatment mares received  $10^7$  CFU of *S. equi* subspecies *zooepidemicus*.

Allantoic fluid was placed immediately on ice after collection, cultured for aerobic bacteria, aliquoted into vials, and processed specifically for each assay before being stored at  $-80^\circ\text{C}$ . Tumor necrosis factor  $\alpha$  was measured with a standard cytolytic bioassay, using tumorigenic fibroblast-like mouse cell line L929 [1]. IL-1 was quantified as previously described as the ability to support growth of the cloned conalbumin-specific mouse T-helper-2 cell line, D10.G4.1 [2]. IL-6 bioassay was performed as previously described using the IL-6 dependent B9 cell line [3]. Prostaglandin  $F_{2\alpha}$  and  $\text{PGE}_2$  were measured by competitive binding radioimmunoassay kits (PGF $_{2\alpha}$ -TRK900 Amersham Pharmacia Biotech, Piscataway, NJ and  $\text{PGE}_2$ -NEK020 NEN Life Science Products, Boston, MA). Both assays were validated in our laboratory. For statistical comparisons, allantoic fluid samples were divided into four time periods. Period 1 included the sample collected before inoculation in experimental mares and on dGa 280 in control mares. Period 2 included the allantoic sample collected 3–4 days after inoculation and the sample from dGa 288–290 in control mares. Period 3 included the sample collected 6–10 days after inoculation and the sample from dGa 296–303 in control mares. Period 4 included the allantoic sample collected within 48 h of delivery in both groups. The effect of time was assessed within control and treatment mares using the Friedman test for each dependent variable. The Wilcoxon Signed Rank Test was then used to compare period means. Significance was based on a  $P$ -value of 0.05.

### 2.1. Experiment 2

The mRNA expression of the pro-inflammatory cytokines IL-8, IL-6, IL-1 $\beta$  and TNF- $\alpha$  were measured in placental tissues of eight mares with experimentally induced placentitis and in seven that delivered normal foals to determine if ascending placentitis was associated with an increase in the expression of cytokines in fetal tissues. Placental tissue was collected from the cervical star region and from one or more grossly normal areas of placenta located at least 10 cm from the grossly abnormal area. Samples were divided into two specimens, with one immediately submersed in liquid nitrogen until assayed for cytokine mRNA expression and the other placed in formalin for histological evaluation. Total RNA was extracted from tissue samples, treated with amplification grade DNase I to remove any traces of genomic DNA contamination and cDNA was synthesized from 1  $\mu\text{g}$  of total RNA. Gene specific primers and internal oligonucleotide probes for IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) were selected from equine specific cytokine cDNA sequences. Relative quantitation of cytokine mRNA expression was performed by real-time PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). All samples were assayed in triplicate with the mean value used for comparisons. To account for variation in the amount and quality of starting material, all the results were normalized to G3PDH expression. Relative quantitation between samples was achieved by comparing their normalized threshold cycles (Ct). The Ct represents the PCR cycle at which an increase in reported fluorescence above the

threshold is detected. Samples without cDNA were included in the amplification reactions as negative controls to determine background fluorescence and check for contamination. The cDNA obtained from Concanavalin A-stimulated equine blood mononuclear cells were used as a positive control. Comparisons of cytokine mRNA expression between the cervical star region and placenta within the same animal were made using the Wilcoxon Signed Rank Test. Comparisons of cytokine mRNA expression between the control and placentitis groups were made using the Mann–Whitney *U*-test.

### 3. Results

Seven of eight experimental mares aborted or delivered non-viable foals between 5 and 27 days after inoculation. One mare delivered a viable foal on dGa 309, 20 days after inoculation. Three of four control mares delivered viable foals at term. The fourth mare aborted after the third allantocentesis at dGa 296 from an iatrogenically induced *Bacillus* infection. Bacteria were recovered only in allantoic fluid samples collected in period 4 of experimental mares. There was an effect of time on the concentrations of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  in allantoic fluid collected from experimental mares with period 4 samples being higher than those of period 1 ( $P < 0.05$ ). Concentrations of PGE<sub>2</sub> rose from a pre-inoculation value of  $108 \pm 29$  pg/ml to  $13,972 \pm 6255$  pg/ml in period 4. Concentration of PGE<sub>2</sub> in allantoic fluid of control mares ranged from 25 to 432 pg/ml. Mean concentration of PGF<sub>2 $\alpha$</sub>  in mares with experimentally induced placentitis rose from  $295.7 \pm 50$  pg/ml before inoculation to  $3241 \pm 1487$  pg/ml by period 4. Concentrations of PGF<sub>2 $\alpha$</sub>  in allantoic fluid of control mares ranged from 105 to 432 pg/ml. There were no differences in the concentrations of IL-1, IL-6 or TNF- $\alpha$  in allantoic fluid between groups or over time. In Experiment 2, mares with experimentally induced placentitis exhibited a suppurative, necrotic placentitis in the region of the cervical star but did not exhibit microscopic lesions in grossly normal placental tissues. Placentas from normal mares exhibited no gross or microscopic lesions. Experimentally infected mares had higher IL-6 ( $P = 0.003$ ) and IL-8 ( $P = 0.009$ ) mRNA expression at the cervical star and significantly higher IL-6 expression ( $P = 0.004$ ) in the placental body than control mares.

### 4. Discussion

From these data we propose the following: in the presence of an ascending bacterial infection, organisms enter the chorioallantois and induce an increase in the expression of pro-inflammatory cytokines in the placental tissue. This results in the release of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  into allantoic fluid which mediate the events that lead to premature labor.

### References

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