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BASIC SCIENCE: OBSTETRICS

Systemic and cerebral inflammatory response to umbilical cord occlusions with worsening acidosis in the ovine fetus

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OBJECTIVE: We hypothesized that repetitive umbilical cord occlusions (UCOs) with worsening acidosis will lead to a fetal inflammatory response.

STUDY DESIGN: Chronically instrumented fetal sheep underwent a series of UCOs until fetal arterial pH decreased to <7.0. Maternal and fetal blood samples were taken for blood gases/pH and plasma interleukin (IL)-1B and IL-6 levels. Animals were euthanized at 24 hours of recovery with brain tissue processed for subsequent measurement of microglia and mast cell counts.

RESULTS: Repetitive UCOs resulted in a severe degree of fetal acidemia. Fetal plasma IL-1B values were increased ~2-fold when measured at maximal fetal acidosis and again at 1-2 hours of recovery. Fetal microglia cells were increased ~2-fold in the white matter and hippocampus, while mast cells were increased ~2-fold in the choroid plexus and now evident in the thalamus when analyzed at 24 hours recovery.

CONCLUSION: Repetitive UCOs leading to severe acidemia in the ovine fetus near term will result in an inflammatory response both systemically and locally within the brain.

Key words: fetal hypoxia, interleukin-Iβ, interleukin-6, mast cells, microglia

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Birth asphyxia with severe fetal acidemia, defined as an umbilical artery pH <7.00, is associated with increased risk for newborn hypoxic-ischemic encephalopathy (HIE), although the majority of these infants will still be without noted complications.[1-4] This indicates that birth asphyxia with resultant brain injury in most instances is multifactorial in basis with gestational age at birth, duration of hypoxic acidemia, fetal/newborn compensatory capacity, and newborn resuscitation also likely to be contributory.[3,5,6]

There is now considerable epidemiologic and clinical evidence that increases in inflammatory cytokines during the course of infection play a contributing role in the increased risk for brain injury, whether intrauterine with chorioamnionitis preterm or at term, or postnatal in the neonate (see review[7]). This has resulted in a number of animal-based studies with the induction of perinatal infection and/or inflammatory response by bacterial products further implicating a contributory role for an increase in fetal inflammatory cytokines along with an increase in inflammatory cells within the brain, in resultant brain injury.[7-9] These animal-based studies furthermore show an interactive effect whereby bacterial endotoxin sensitizes the immature brain to hypoxic-ischemic injury indicating that infection and hypoxic acidemia may have a synergistic role in causing fetal brain injury.[7,10,11] There is also considerable clinical and experimental evidence that increases in inflammatory mediators play a contributory role in the pathogenesis of newborn HIE in the absence of overt infection, although most cases of histopathologic chorioamnionitis will be subclinical especially at term.[7,12-14] In addition, hypoxia and hypoperfusion both lead to increases in cytokine expression and/or production within the placenta[15,16] supporting the contention that reduced uterine or umbilical blood flow with contractions through labor might lead to an increase in inflammatory cytokines as well as worsening fetal acidosis.

Variable-type fetal heart rate (FHR) decelerations due to umbilical cord compression with acute reduction in fetal oxygenation are the most common nonreassuring FHR pattern observed intrapartum.[17] Although these short-term hypoxic episodes are generally well tolerated, when more frequent and/or severe they have been associated with an increased incidence of neonatal acidosis, low Apgar scores, and nuchal cord involvement at the time of delivery.[18,19] We have therefore used the chronically catheterized ovine fetus near term to test

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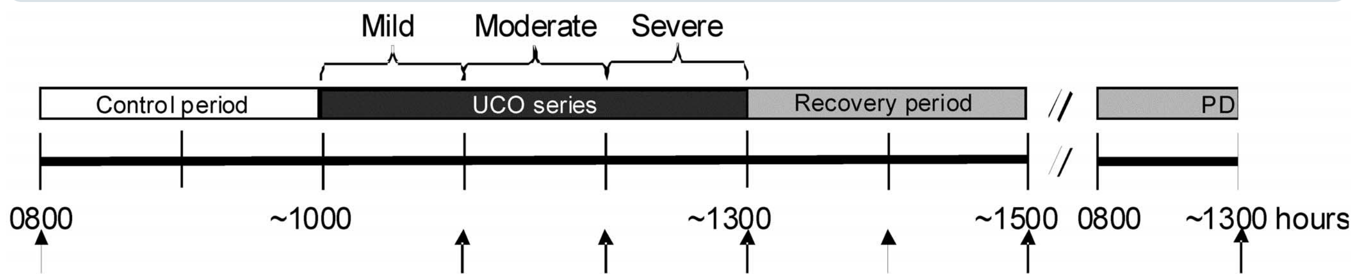
FIGURE 1
Experimental protocol



Animals were studied through 1- to 2-hour control period; ~3 hours of mild, moderate, and severe repetitive umbilical cord occlusions (UCOs); and then recovered for ~24 hours. *Arrows* indicate 3-mL fetal and/or maternal blood samples.

PD, putdown.

Prout. Inflammation with fetal asphyxia. Am J Obstet Gynecol 2009.

the hypothesis that repetitive cord occlusions with worsening acidosis as might be seen clinically during labor will lead to an inflammatory response both systemically and locally within the brain. The proinflammatory cytokines interleukin (IL)-1B and IL-6 have been determined as measures of systemic inflammation because these cytokines play a prominent regulatory role in the inflammatory response and have been shown to increase as part of the fetal/neonatal inflammatory response to infection and with HIE.[7,12-14] The distribution of microglia and mast cells within the brain have been determined as measures of local inflammation because these cells also play a prominent role in the inflammatory response and likewise have been shown to increase with fetal/neonatal infection and/or hypoxia.[7-9,20]

Materials and Methods

Surgical preparation

Ten near-term (125 ± 1 days' gestation) fetal sheep of mixed breed were surgically instrumented (term = 145 days). The anesthetic and surgical procedures and postoperative care of the animals have previously been described.[21] Briefly, using sterile technique under general anesthesia, the upper body of the fetus and proximal portion of the umbilical cord were exteriorized through an incision in the uterine wall. Polyvinyl catheters (Bolab, Lake Havasu City, AZ) were placed in the right and left brachiocephalic arteries, and the right brachiocephalic vein. Stainless steel electrodes were implanted biparietally on the dura for the recording of electrocortical activity and over the sternum for recording electrocardiographic (ECG) activity. An inflatable silicone occluder cuff (OCHD16; In Vivo Metric, Healdsburg, CA) was positioned around the proximal portion of the umbilical cord and secured to the abdominal skin. Once the fetus was returned to the uterus, a catheter was placed in the amniotic fluid cavity and subsequently in the maternal femoral vein.

Animals were allowed a 3- to 4-day postoperative period before experimentation, during which antibiotics were given and catheters were flushed with heparinized saline to maintain patency. Animal care followed the guidelines of the Canadian Council on Animal Care and was approved by the University of Western Ontario Council on Animal Care.

Experimental protocol

Animals were studied through a 1- to 2-hour control period and an experimental period of repetitive umbilical cord occlusions (UCOs) with worsening acidemia, and were then allowed to recover overnight (Figure 1). A computerized data acquisition system was used to record pressures in the fetal brachiocephalic artery and amniotic cavity, and the electrical signals for electrocortical and ECG activities, which were monitored continuously through the control and experimental periods, and first 2 hours of the recovery period (Chart 5 for Windows; AD Instruments Pty Ltd). AQ: 4

After the baseline control period that began at ~0800 hours, repetitive UCOs were performed with increasing severity until severe fetal acidemia was detected (arterial pH <7.00), at which time the UCOs were stopped. UCO was induced by complete inflation of the occluder cuff with ~5 mL of saline solution that was previously determined by visual inspection and testing at the time of surgery. During the first hour a mild UCO series was performed consisting of cord occlusion for 1-minute duration every 5 minutes. During the second hour a moderate UCO series was performed consisting of cord occlusion for 1-minute duration every 3 minutes. During the third hour a severe UCO series was performed consisting of cord occlusion for 1-minute duration every 2 minutes and this series was continued until the targeted fetal arterial pH was attained. Following the mild and moderate UCO series a 5- to 10-minute period with no UCO was undertaken, during which fetal arterial blood was sampled and arterial blood pressure, ECOG, and ECG data were recorded in the absence of FHR decelerations. After attaining the targeted fetal arterial pH <7.00 and stopping the repetitive UCOs, animals were allowed to recover for ~24 hours. AQ: 5 AQ: 6 F1

Fetal arterial blood samples were obtained during the baseline control period (3 mL), at the end of the first UCO of each UCO series (1 mL), and ~5 minutes after each UCO series (3 mL). In ad-

dition, fetal arterial blood samples were obtained between UCOs at ~20 and 40 minutes of the moderate and severe UCO series (1 mL), and at 1, 2, and 24 hours of recovery (3 mL). Maternal venous blood samples were also obtained during the baseline control period, and at 1 and 24 hours of recovery (3 mL). All fetal blood samples were analyzed for blood gas values, pH, glucose, and lactate with an ABL-725 blood gas analyzer (Radiometer Medical, Copenhagen, Denmark) with temperature corrected to 39.0°C. Fetal and maternal 3-mL blood samples at selected time points (Table) were spun at 4°C (4 minutes, 4000*g* force; Beckman TJ-6; Fullerton, CA) and the plasma decanted and stored at –80°C for subsequent cytokine analysis.

After the 24-hour recovery blood sample, the ewe and the fetus were killed by an overdose of barbiturate (30 mg of sodium pentobarbital intravenously; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and a postmortem examination was carried out during which time fetal gender and weight were determined, and the location and function of the umbilical cord occluder cuff were confirmed. The fetal brain was then perfusion fixed with 500 mL of cold saline followed by 500 mL of 4% paraformaldehyde and processed for histochemical analysis as we have previously reported.[22] To obtain brain tissue from control animals for comparative purposes to that of the repetitive UCO animals, 2 noninstrumented twins of instrumented experimental group animals and 4 instrumented control animals from a separate study were used. These latter animals were similarly instrumented and of the same gestational age as the repetitive UCO animals and all animals underwent the same perfusion-fixation procedure and brain tissue processing for histochemical study.

Plasma cytokine and tissue histochemical analysis

An enzyme-linked immunosorbent assay was used to analyze in duplicate the concentrations of IL-1β and IL-6 in fetal arterial and maternal venous plasma samples. IL-1β and IL-6 standards were purchased from the University of Melbourne, Center of Animal Biotechnology (Melbourne, Australia). Mouse antiovine IL-1β (MAB 1001) and IL-6 (MAB 1004) monoclonal antibodies and rabbit antiovine IL-1β (AB 1838) and IL-6 (AB 1889) polyclonal antibodies were purchased from Chemicon International (Temecula, CA). Separate 96-well plates were coated with mouse monoclonal ovine IL-1β or IL-6 antibody (1:200, in 0.1 mol/L of $NaCO_3$, pH to 9.6) and incubated overnight at 4°C. The following day, plates were washed 3 times with wash solution (1X phosphate-buffered saline [PBS] with 0.05% Tween, pH to 7.4) to remove excess monoclonal antibody. Plates were then blocked with assay diluent (555213, BD OptEIA, BD Biosciences) at room temperature for 1 hour. Wells were then rinsed 3 times with the wash solution followed by aliquoting standards (40,000-156 pg/mL and blanks) and samples, and incubation on the shaker at room temperature for 2 hours. Subsequently, wells were rinsed 3 times with washing solution and the appropriate rabbit antiovine polyclonal antibody (IL-1β or IL-6, 1:500) was added to each well and incubated on the shaker for 1 hour. Following ≥5 washes, HRP-donkey anti-rabbit IgG (AP182p, 1:10,000; Chemicon International) was added to each well and incubated on the shaker for 1 hour. The wells were then washed 7 times with wash solution to remove all unbound secondary antibody, followed by the addition and 30-minute incubation with substrate solution (51-2606KC and 51-2607KC; BD Biosciences) in the dark. Stop solution (1N H_2SO_4) was applied and each well was read using a spectrophotometer at 450 nm, with 575-nm wavelength correction.

The presence of microglia in brain tissue was determined by avidin-biotin-peroxidase complex enhanced immunohistochemistry (Vectastain Elite; Vector Laboratories, Burlingame, CA). To reduce staining variability, all immunohistochemistry was performed on the same day with the same batch of antibody and solutions. Tissue sections were deparaffinized in 3 sequential xylene baths for 5 minutes each and subsequently rehydrated in a series of alcohol baths (100%, 90%, 70%) each lasting 2 minutes followed by a 5-minute rinse in tap water and equilibration in PBS. Antigen retrieval was performed by incubation of the sections in boiling citrate buffer (2.1 g of citric acid/1 L of water; pH 6.0 with concentrated NaOH) in a steamer for 30 minutes followed by a 35-minute cooling period in the citrate buffer outside of the steamer and a 5-minute rinse in tap water. Sections were then washed 3 times in PBS before and after endogenous peroxidases were quenched by a 10-minute bath in 3% hydrogen peroxide in PBS. Avidin-biotin blocking was performed with a 15-minute incubation first with avidin followed by biotin. Nonspecific protein binding was blocked with a 10-minute incubation in background sniper blocking serum (Biosource Medical, Concord, CA). Sections were then incubated with an anti-ionized calcium-binding adaptor molecule (IBA)1 rabbit polyclonal antibody (1:500; Wako Industries, Richmond, VA) diluted in Dako diluent solution (Dako Cytoma-

TABLE
Plasma cytokine measurements (pg/mL)

Fetal				
IL-1β	525 ± 96	1068 ± 167[a]	967 ± 152[a]	640 ± 143
IL-6	429 ± 50	460 ± 78	446 ± 58	440 ± 102
Maternal				
IL-1β	379 ± 64		294 ± 64	
IL-6	563 ± 160		682 ± 243	

IL, interleukin.
Data are presented as means ± SEM.
[a] $P < .05$ vs respective baseline values.
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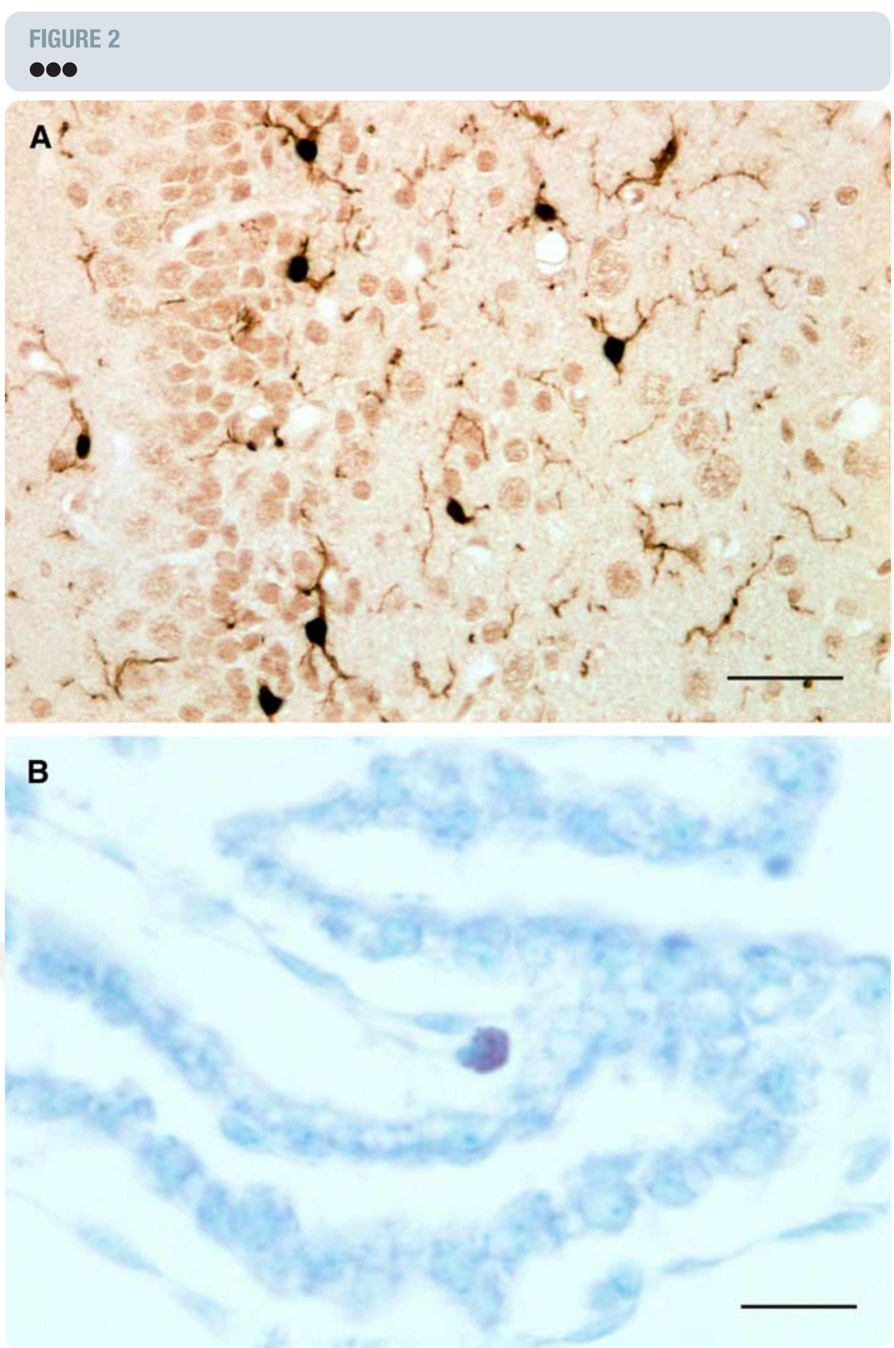
tion, Carpinteria, CA) overnight at 4°C, which has been reported to be a robust marker for microglia in human and animal studies.[23,24] Sections were subsequently rinsed 3 times for 5 minutes in PBS and then incubated with secondary antibody (1:200, biotinylated antirabbit immunoglobulin G; Vector Laboratories) at room temperature for 30 minutes and rinsed as described earlier. Sections were then incubated with streptavidin/biotin/peroxidase/reagent (Vectastain ABC Elite; Vector Laboratories Inc) at room temperature for 45 minutes. The detection of bound antibody was obtained with a 2-minute incubation in Cardassian DAB Chromogen (Biocare Medical, Concord, CA) at room temperature. Sections were rinsed with running tap water for 5 minutes, then dehydrated in 5 brief alcohol baths of increasing concentration (1 bath in 70% alcohol, 2 baths in 90%, and 2 baths in absolute alcohol), followed by 3 xylene baths of 5 minutes each before being cover slipped in Permount (Fisher Scientific, Ottawa, Ontario, Canada). To demonstrate nonspecific binding, additional negative control sections were processed as described, with the exception that the primary antibody was omitted.

The presence of mast cells in brain tissue was determined using histologic and morphologic assessment techniques. Tissue sections were deparaffinized in 3 sequential xylene baths for 5 minutes each and subsequently rehydrated in a series of alcohol baths (100%, 90%, 70%) each lasting 2 minutes followed by a 5-minute rinse in tap water and equilibration in PBS. Sections were then stained in 0.1 mol/L of HCL with toluidine blue (pH = 2) for 10 minutes and then rinsed with running tap water for 5 minutes. Sections were quickly rinsed in acetic acid, then dehydrated in 5 brief alcohol baths of increasing concentration (1 bath in 70% alcohol, 2 baths in 90%, and 2 baths in absolute alcohol), followed by 3 xylene baths of 5 minutes each before being cover slipped in Permount.

Brain regions that were selected from each animal for analysis were taken from a coronal section of blocked cerebral hemisphere tissue at the level of the mamillary bodies and included the parasagittal and convexity cerebral gray matter and leptomeninges, periventricular white matter, thalamus, choroid plexus, and the combined CA2 and CA3 regions of the hippocampus. Each of the gray matter regions was further divided into subregions combining layers 1, 2, and 3 and layers 4, 5, and 6. After showing no significant difference between these subregions, all layers were combined to represent the gray matter. Image analysis

FIGURE 2
●●●



Photomicrographs showing **A**, microglia in hippocampus identified by anti-ionized calcium-binding adaptor molecule 1 contiguous cytoplasmic staining (bar = 20 μm) and **B**, mast cell in choroid plexus identified by toluidine blue staining and characteristic morphology with presence of large metachromatic secretory granules filling cytoplasm and unilobular ovoid nucleus (bar = 10 μm).

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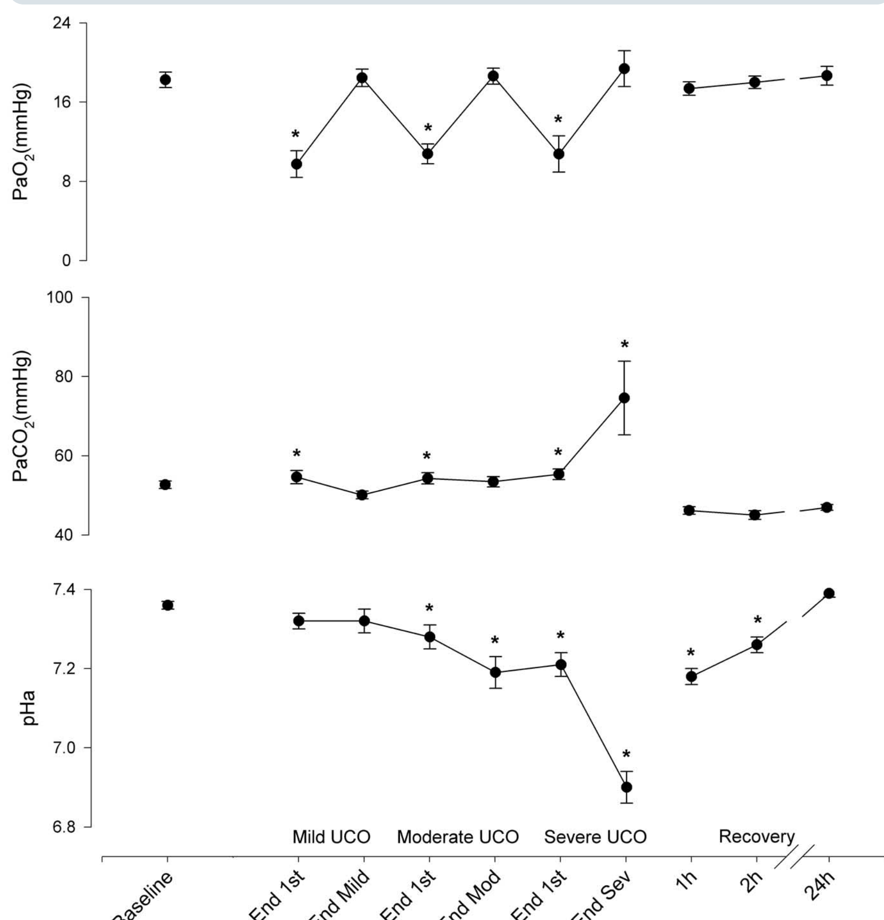
FIGURE 3



Fetal arterial blood gases and pH at baseline; at end of first umbilical cord occlusions (UCOs) and series of UCOs, for each of mild, moderate, and severe (sev) UCO series; and during recovery. Values are means ± SEM. *$P < .05$ compared with respective baseline values.

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was performed with a transmitted light microscope (Leica DMRB; Leica-Microsystems, Wetzler, Germany) at ×40 magnification. Positive microglia cell immunostaining was quantified with an image analysis program (Image Pro Plus 6.0; Media Cybernetics, Silver Spring, MD). The image analysis system was first calibrated for the magnification settings that were used, and thresholds were established to provide even lighting and no background signal. Six high-power field (HPF) photomicrographs (HPF area = 7 cm^2) per brain region/subregion per animal were collected as a 24-bit RGB color modeled image. The same illumination setting was applied to all images for all of the brain regions, therefore allowing for comparison within each brain region (ie, control vs repetitive cord occlusion animal groups), and between brain regions (ie, gray matter vs white matter). For the microglia analysis, and using the Image Pro Plus' RGB color range selection tool, color sampling of positive DAB-stained areas were obtained from multiple brain regions of control and UCO animals, and tested for specificity against the negative control. Appropriate ranges of color were selected showing positive contiguous cytoplasmic staining as criteria for microglia cell count scoring, which were then applied uniformly to calibrated images for all brain regions (Figure 2). Scoring was performed in a blinded fashion to experimental groups. For mast cell analysis, scoring was performed manually based on positive stain and characteristic morphology with the presence of large metachromatic secretory granules filling the cytoplasm due to the presence of sulfonated proteoglycans such as heparin, and a unilobular ovoid nucleus (Figure 2). Scoring was again performed in a blinded fashion to experimental groups.

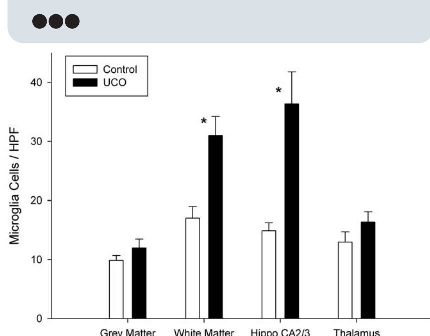
Data analysis

Blood gas and pH measurements in response to repetitive cord occlusion were compared to the corresponding baseline values by 1-way repeated measures analysis of variance with Student-Newman-Keuls post hoc correction. For the enzyme-linked immunosorbent assay cytokine measurements analysis was performed using Wilcoxon (signed rank) test with Holm correction of *P* values for multiple comparisons. For analysis of microglia and mast cell numbers across brain regions and between control and cord occlusion animal groups, a 2-way repeated measures analysis of variance was performed with Holm-Sidak post hoc correction. Correlation analyses were performed using Spearman correlation coefficient (SigmaStat; Systat Software Inc, San Jose, CA). All values are expressed as means ± SEM. Statistical significance was assumed for $P < .05$. The cardiovascular, electrocortical, and metabolic results from these animals have, or will be, reported separately.[25]

RESULTS

Repetitive cord occlusion insults as studied resulted in fetal arterial blood gas and pH changes with each cord occlusion and cumulative changes over the course of study (Figure 3). UCO of 1-minute duration produced a large decrease in fetal arterial P_{O_2} and to a similar extent as measured at the end of the first UCO of each of the mild, moderate, and severe UCO series from a baseline value of 18.3 ± 0.8-10.4 ± 0.8 mm Hg on average ($P < .05$). UCO of 1-minute duration also produced an increase in fetal arterial P_{CO_2} and to a similar extent as measured

FIGURE 4



Bar graph of microglia immunoreactivity expressed as number of identified microglia cells staining ionized calcium-binding adaptor molecule positive (see "Methods")/high-power field (HPF) in different regions of control (*open bars*) and umbilical cord occlusion (UCO) (*shaded bars*) group animals. Values are means ± SEM. Hippo = hippocampus. **P* < .05.

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from a baseline value of 52.7 ± 0.9-57.5 ± 0.9 mm Hg on average ($P < .05$). Fetal arterial pH was variably decreased as measured at the end of the first UCO of each of the mild, moderate, and severe UCO series, from the baseline value of 7.36 ± 0.01, but with a cumulative decrease in pH after each of the UCO series also evident.

Fetal arterial Po_2 values were not significantly different from that of the initial baseline value of 18.3 ± 0.8 mm Hg when again measured at 5 minutes following completion of each of the mild, moderate, and severe UCO series, indicating that fetal oxygenation returned to preocclusion levels after the occluder release with no cumulative change in this measurement over successive occlusions throughout the study (Figure 3). Pco_2 values were likewise similar to the initial baseline value of 52.7 ± 0.9 mm Hg when again measured at 5 minutes following completion of the mild and moderate UCO series. However, these values were significantly increased when measured following completion of the severe UCO series at 77.3 ± 9.3 mm Hg ($P < .05$), and indicating a respiratory component to the maximal fetal acidosis at this time. Fetal arterial pH values showed a progressive decrease from that of the initial baseline value of 7.36 ± 0.01 when again measured following completion of the mild, moderate, and severe UCO series at 7.32 ± 0.03, 7.19 ± 0.04 ($P < .05$), and 6.90 ± 0.05 ($P < .05$), respectively, and indicating a worsening fetal acidosis that was greatest during the severe UCO series (Figure 3). Fetal arterial base excess values likewise showed a progressive decrease from that of the initial baseline value of 3.7 ± 0.5 mmol/L when measured following completion of the mild, moderate, and severe UCO series at 0.7 ± 1.4, –6.0 ± 2.2 ($P < .05$), and –16.6 ± 1.0 mmol/L ($P < .05$), respectively, and indicating a predominant metabolic component to the worsening acidosis throughout the study. Of note, 2 animals reached the target pH < 7.00 during the moderate UCO series, whereas the remaining 8 animals took between 20 and 100 minutes during the severe UCO series to reach the target pH.

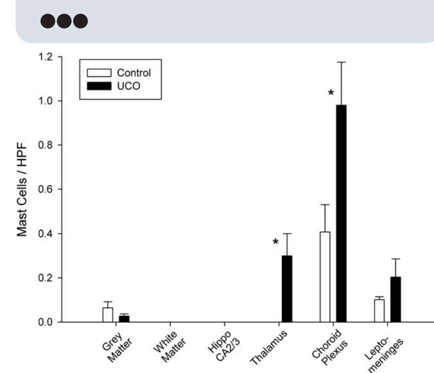
The animal with the lowest arterial pH at 6.64 died shortly after stopping the repetitive cord occlusion insults. In the remaining 9 animals Pco_2 values were back to baseline when again measured at 1 hour of recovery, however, pH and base excess values were still significantly decreased at 7.26 ± 0.02 and –5.4 ± 0.7 mmol/L, respectively, from that at baseline when measured at 2 hours of recovery (both $P < .05$) (Figure 3). When again measured at 24 hours of recovery, pH and base excess values were now back to baseline.

Fetal arterial Po_2, Pco_2, and pH values for the 4 instrumented control animals that were used for brain tissue comparisons measured 19.8 ± 1.5 mm Hg, 50.1 ± 1.9 mm Hg, and 7.36 ± 0.01, respectively, which were similar to that of the initial baseline values for the 10 UCO experimental animals.

Plasma cytokine measurements

Plasma cytokine measurements as obtained at selected time points from fetal arterial and maternal venous blood sampling are shown in the Table. Fetal IL-1β values were significantly increased from the baseline value of 525 ± 96 pg/mL when again measured at the end of the severe UCO series when fetal acidosis was maximal and at 1-2 hours of recovery, by ~100% and ~80%, respectively, (both $P < .05$). Although fetal IL-1β values were still increased by ~20% when measured at 24 hours of recovery, this was no longer significant. Conversely, fetal IL-6 values remained little changed from that at baseline when again measured at the end of the severe UCO series and at 1-2 and 24 hours of recovery, and overall averaged 439 ± 68 pg/mL. Maternal IL-1β and IL-6 values also remained little changed from that at baseline when again measured at 1-2 hours of recovery and overall averaged 337 ± 64 and 622 ± 189 pg/mL, respectively.

FIGURE 5



Bar graph of mast cell distribution expressed as number of identified mast cells staining toluidine blue positive and with characteristic morphology (see "Methods")/high-power field (HPF) in different regions of control (*open bars*) and umbilical cord occlusion (UCO) (*shaded bars*) group animals. Values are means ± SEM. **P* < .05.

Hippo, hippocampus.

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Histochemical scoring for microglia and mast cells

Microglia immunoreactivity was analyzed at 24 hours recovery as a measure of local inflammatory response within the brain. In the control group animals the average microglia cell count across all brain regions was 14 ± 2 cells/HPF and this was not significantly different regardless of the region (Figure 4). In the fetuses subjected to repetitive UCOs, the microglia cell counts remained unchanged compared to their respective control levels in the cerebral gray matter

and the thalamus (Figure 4). However, the microglia cell count was significantly increased to 31 ± 5 and 36 ± 12 cells/HPF in the white matter and hippocampus, respectively, when compared to control levels (both $P < .05$) (Figure 4).

Mast cell distribution was analyzed at 24 hours recovery as a second measure of the local inflammatory response within the brain. In the control group animals the mast cell count across the brain regions overall was extremely low ranging from 0.4 ± 0.1 cells/HPF in the choroid plexus to being absent in the white matter, hippocampus, and thalamus, although none of these regional differences were significant (Figure 5). In the UCO group animals the mast cell count, although little changed in the gray matter, white matter, hippocampus, and leptomeninges, was significantly increased in the thalamus and choroid plexus to 0.3 ± 0.1 and 1.0 ± 0.2 cells/HPF, respectively, when compared to control levels (both $P < .05$) (Figure 5).

Individual animal findings for those exposed to repetitive UCO were correlated to further assess the relationship of the degree of maximal acidosis and time to reach such to the cytokine and inflammatory cell outcomes. Although the degree of maximal fetal acidosis attained whether using pH or BE did not relate to the associated IL-1β findings, the total duration of repetitive UCOs did show a positive correlation to the increase in IL-1β at this time, r = 0.66, P = .05. Likewise, the total duration of repetitive UCOs was highly correlated to the white matter/hippocampus microglia cells/HPF, r = 0.90, P = .02. However, there was no correlation found between the IL-1β findings at maximal fetal acidosis and either the microglia or mast cell findings as studied.

COMMENT

In this study, we have used the chronically catheterized ovine fetus near term subjected to repetitive UCOs of increasing frequency thereby leading to worsening acidosis as might be seen clinically during labor. All animals attained the targeted arterial pH <7.00 and thereby a severe degree of fetal acidemia that was predominantly metabolic in nature with base excess values down to –16 mmol/L on average, although the total duration of repetitive UCOs required was variable ranging from 100-220 minutes.

Fetal plasma IL-1B values were increased by ~2-fold when measured at maximal fetal acidosis and again at 1-2 hours of recovery, indicating that a systemic inflammatory response is initiated with repetitive UCOs leading to severe acidemia. Although the cellular origin and stimulus for the increase in this proinflammatory cytokine are unknown, in vitro study of the isolated perfused human placenta has demonstrated increased production of inflammatory cytokines in response to hypoperfusion[15] as would be seen with repetitive UCOs. In addition, hypoxia induces expression of several cytokines in the placenta including IL-1B[16] and hypoxic-ischemic tissue response/injury will result in cytokine production by leukocytes, endothelium, and other cells through autocrine and paracrine mechanisms.[7,12-14] Fetal IL-1B values were back toward baseline levels when measured at 24 hours of recovery, which is to be expected with blood gas and pH values now normalized, and given the short half-life of cytokines and need for repeated stimuli for continued production.[7] Conversely, fetal IL-6 values remained relatively unchanged as studied, which is somewhat surprising since IL-6 is a major mediator of the acute-phase response to tissue injury[7] and has been shown to increase in the cord blood of newborn infants with perinatal asphyxia.[13,14] Although this may relate to species-specific differences, it is also possible that IL-6 has been increased, but at a later time point than 1-2 hours of recovery because IL-1B is known to stimulate expression of IL-6.[7] Maternal IL-1B and IL-6 values also remained unchanged, indicating that if the production of these cytokines is in fact increased within the placenta, then they are not released into the maternal circulation or the amount is insufficient to impact on maternal levels.

Fetal microglia cell counts were increased by ~2- and 2.5-fold in the white matter and hippocampus, respectively, when analyzed at 24 hours recovery, indicating that a local inflammatory response within the brain is also initiated with repetitive UCOs leading to severe acidemia. Anti-IBA1, as used in the current study, is an affinity purified rabbit polyclonal antibody raised against IBA1, a calcium-binding protein that is specifically expressed in microglia/macrophages and up-regulated during the activation of these cells.[23,24] As such, the present findings likely indicate an increase in activated microglia in these brain regions. This finding would be expected with evolving inflammation in response to a noxious stimulus, in this case hypoxic ischemia as previously shown postnatally.[26] It is of note that the increase in brain microglia was in the white matter, which is also seen in the ovine fetus after chronic endotoxin exposure and relates to the degree of white matter injury,[8] and in the hippocampus, a brain region that is particularly vulnerable to neuronal loss in response to hypoxic-ischemic insult in the ovine fetus near term.[27] Furthermore, the finding of the increased microglia cell counts at 24 hours of recovery and before the time of expected brain injury with overt cell death from induced hypoxic asphyxia[28] would implicate a role for microglial activation in the mechanisms of injury.

Mast cells are now widely recognized as key cellular components of inflammatory processes, storing a range of biologic mediators and capable of making additional ones when stimulated.[7,20] Most study in the developing brain has been in newborn rat pups where they are initially concentrated in the leptomeninges and choroid plexus, later entering the brain parenchyma and predominantly the thalamus, along penetrating blood vessels.[20,29] Notably, after hypoxic-ischemic insult there is increased migration of mast cells into the thalamus, cingulate cortex, and hippocampus, and evident degranulation,[20] which supports the contention that mast cells might also play a contributory role in the pathogenesis of newborn HIE. To our knowledge, this is the first study in the developing ovine fetal brain where the mast cells, although sparse, again appeared to be concentrated in the choroid plexus and

leptomeninges in the control group animals. At 24 hours after repetitive UCOs with severe fetal acidemia, mast cells were increased in the choroid plexus and evident in the thalamus, findings similar to that in the newborn rat pup[20] and further supporting a causal role for mast cells in newborn HIE.

In this study, we have determined that repetitive UCOs leading to severe acidemia in the ovine fetus near term will result in an inflammatory response both systemically with an increase in plasma IL-1B levels at the time of maximal acidosis, and locally within the brain with an increase in microglia and mast cells as measured 24 hours thereafter. Although there was no correlation found between the IL-1B findings and either the microglia or mast cell findings as studied, it is certainly possible that the increase in circulating cytokines has modulated the local inflammatory response within the brain given their ability to increase blood-brain permeability to macrophages and other inflammatory mediators[7,8] and the time course for these events. There is now considerable evidence that inflammation both systemically and locally within the brain in response to perinatal infection[7-11] and hypoxic asphyxia at birth[12-14] plays a causal role in brain injury of the newborn. The present findings in an animal model relevant to human labor with repetitive UCOs leading to severe fetal acidemia and in the absence of infection, adds to this evidence. Most importantly, fetal (neonatal) inflammation, whether triggered by hypoxic acidemia alone during labor, or in association with related events such as placental hypoperfusion or a degree of chorioamnionitis, should be considered as a cofactor in assessing risk for brain injury with severe acidemia at birth. It is of note that the total duration of repetitive UCOs as herein studied was well correlated to the systemic and brain inflammatory responses, suggesting that the duration of any inflammatory stimulus during human labor may be more important in determining outcome than the actual intensity of the stimulus. It is also of note that UCO-variable FHR decelerations relate to chemoreceptor-mediated vagal activation, which has been shown in adult studies to modify inflammatory responses through cholinergic anti-inflammatory pathway mechanisms.[30] As such, the degree of vagal activation with repetitive UCOs and worsening acidemia may also impact on the cumulative inflammatory response over time and warrants further study. ■

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000 **Systemic and cerebral inflammatory response to umbilical cord occlusions with worsening acidosis in the ovine fetus**

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Repetitive umbilical cord occlusions leading to severe acidemia in the ovine fetus near term will result in an inflammatory response both systemically and locally within the brain.