The Impact of Intermittent Umbilical Cord Occlusions on the Inflammatory Response in Pre-Term Fetal Sheep

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Abstract

Fetal hypoxic episodes may occur antepartum with the potential to induce systemic and cerebral inflammatory responses thereby contributing to brain injury. We hypothesized that intermittent umbilical cord occlusions (UCOs) of sufficient severity but without cumulative acidosis will lead to a fetal inflammatory response. Thirty-one chronically instrumented fetal sheep at ~0.85 of gestation underwent four consecutive days of hourly UCOs from one to three minutes duration for six hours each day. Maternal and fetal blood samples were taken for blood gases/pH and plasma interleukin (IL)-1β and IL-6 levels. Animals were euthanized at the end of experimental study with brain tissue processed for subsequent counting of microglia and mast cells. Intermittent UCOs resulted in transitory fetal hypoxemia with associated acidemia which progressively worsened the longer umbilical blood flow was occluded, but with no cumulative blood gas or pH changes over the four days of study. Fetal arterial IL-1β and IL-6 values showed no significant change regardless of the severity of the UCOs, nor was there any evident impact on the microglia and mast cell counts for any of the brain regions studied. Accordingly, intermittently UCOs of up to three minutes duration with severe, but limited fetal hypoxemia and no cumulative acidemia, do not result in either a systemic or brain inflammatory response in the pre-term ovine fetus. However, fetal IL-1β and IL-6 values were found to be well correlated with corresponding maternal values supporting the placenta as a primary source for these cytokines with related secretion into both circulations. Female fetuses were also found to have higher IL-1β levels than males, indicating that gender may impact on the fetal inflammatory response to various stimuli.

Variable fetal heart rate (FHR) decelerations suggesting umbilical cord compression and resultant fetal hypoxemia are seen clinically in 2% to 10% of antenatal FHR recordings near-term [9–11] and have been associated with increased risk for muckle cord at delivery and adverse neonatal outcome. In this regard, the association of infants with a symptomatic and/or tight nuchal cord at delivery and adverse neonatal outcome. In this regard, the association of infants with a symptomatic and/or tight nuchal cord at delivery and the later development of subclinical neurodevelopmental deficits [12] and cerebral palsy [13] implicate a role for chronic intermittent UCO insults antenatally in longer-term injury to the brain. Moreover, we have previously shown in the ovine fetus that intermittent UCO over a four day period does lead to a low level of necrotic appearing cells in the gray matter [14] and a marginal increase in apoptotic appearing cells in the hippocampus [15].

We have therefore used the chronically catheterized ovine fetus to test the hypothesis that intermittent UCOs of sufficient severity but without cumulative acidosis to ensure survival and thereby relevance to antenatal study, will also lead to an inflammatory response. The pro-inflammatory cytokines IL-1β and IL-6 have been determined as measures of systemic inflammation since these cytokines play a prominent regulatory role in the perinatal inflammatory response to infection and with
Experimental Procedure

and abdominal wall incisions were sutured in layers and catheters
were placed in the amniotic fluid cavity and subsequently
around the proximal portion of the umbilical cord and secured to
the right brachiocephalic vein. An inflatable silicone occluder cuff
AZ) were placed in the right and left brachiocephalic arteries, and
in the uterine wall. Polyvinyl catheters (Bolab, Lake Havasu City,
Ohio) were inserted into the right and left brachiocephalic
arteries and given reports whereby fetal-placental immune
responses may be gender based as in later life [22–25].

Methods

Ethics Statement

This study was carried out in strict accordance with the
recommendations for the care and use of laboratory animals by
the Canadian Council on Animal Care. The protocol was
approved by the Committee on the Ethics of Animal Experiments
of the University of Western Ontario (Permit Number: 2005-061-
09; ‘Fetal Brain Development: The Impact of Acute and Chronic
Hypoxia’).

Surgical Preparation

Thirty-one pre-term (113–117 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 [26]. Briefly, using sterile
technique under general anesthesia (1 g thiopental sodium in
solution intravenously [IV] for induction; Abbott Laboratories
Ltd., Montreal, Canada; followed by 1% to 1.5% halothane in O2
for maintenance), a midline incision was made in the lower
abdominal wall, and the uterus was palpated to determine fetal
number and position. 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. 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. Antibiotics were administered intra-
operatively to the mother, (0.2 g trimethoprim and 1.2 g
sulfadoxine, Schering Canada Inc., Pointe-Claire, Canada), fetus
and amniotic cavity (1 million IU penicillin G sodium, Pharma-
cetical Partners of Canada, Richmond Hill, Canada). The uterus
and abdominal wall incisions were sutured in layers and catheters
exteriorized through the maternal flank and secured to the back of
the ewe in a plastic pouch.

Animals were allowed a 3–4 day postoperative period prior to
experimentation, during which the antibiotic administration was
continued. Arterial blood was sampled each day for evaluation of
fetal condition 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 Procedure

Animals were studied over a four day experimental period of
intermittent UCOs of varying duration. A computerized data
acquisition system was used to record pressures in the fetal
brachiocephalic artery and amniotic cavity, which were monitored
continuously through the 4 day study. After a 2 hour baseline
control period which began at ~0800, six intermittent UCOs were
performed over a six hour period, followed by a 1 hour recovery
period on each of the four experimental days. UCO was induced
by complete inflation of the occluder cuff with ~5 mL saline
solution which was previously determined by visual inspection and
testing at the time of surgery. Animals were arbitrarily placed into
either control, mild, moderate or severe UCO groups. The control
group (n = 9) received no occlusions, while the mild UCO group
(n = 6) received cord occlusions of 1 minute duration every hour,
the moderate UCO group (n = 8) received cord occlusions of 2
minute duration every hour, and the severe UCO group (n = 8)
received cord occlusions of 3 minute duration every hour.
Maternal venous and fetal arterial blood samples (3 mL) were
obtained five minutes before the first cord occlusion on days one
and four, and five minutes after the last cord occlusion on days one
and four. Fetal arterial blood samples (1 mL) were additionally
obtained five minutes before, at the end of, and five minutes after
the first and last cord occlusions on days one and four. Maternal
and fetal 3 mL blood samples were immediately 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. Fetal 1 mL blood samples were analyzed for blood gas
values and pH with an ABL-725 blood gas analyzer (Radiometer,
Copenhagen, Denmark) with temperature corrected to 39°C.

After the 1 hour recovery period on day four, the ewe and fetus
were killed with an overdose of barbiturate (30 mg pentobarbital
sodium, Fatal-Plus; Vortech Pharmaceuticals, Dearborn, MI) and
a post mortem was carried out during which 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 [14].

Plasma Cytokine and Tissue Histochemical Analysis

An ELISA was used to analyse in duplicate the concentrations
of IL-1β and IL-6 in fetal arterial and maternal venous plasma
samples as we have previously reported [1]. IL-1β and IL-6
standards were purchased from the University of Melbourne,
Centre of Animal Biotechnology, Melbourne, Australia. Mouse
anti-ovine IL-1β (MAB 1001) and IL-6 (MAB 1004) antibodies
and rabbit anti-ovine IL-1β (AB 1838) and IL-6 (AB 1899)
polyclonal antibodies were purchased from Chemicon Interna-
tional, Temecula, CA.

The presence of microglia in brain tissue was determined by
avidin-biotin-peroxidase complex enhanced immunohistochemistry
(Vectastain Elite; Vector Laboratories, Burlingame, CA) as we
have previously reported [1]. To reduce staining variability, all
immunohistochemistry was performed on the same day with
the same batch of antibody and solutions. Tissue sections were
incubated with an anti-IgA1 rabbit polyclonal antibody (1:500,
Wako Industries, Richmond, VA) which has been reported to be
a robust marker for microglia in human and animal studies
[27,28] with detection of bound antibody obtained following
incubation in Cardassian DAB Chromogen (Biocare Medical,
Concord, CA). The presence of mast cells in brain tissue was
determined using histological and morphological assessment
techniques after tissue sections were stained in 0.1 M HCL with
toluidine blue [1].

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 leptomene-
ingen, 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 sub-regions 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 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 cm²) per brain region/subregion per animal were collected as a 24 bit RGB colour modeled image. The same illumination setting was applied

Figure 1. Photomicrographs taken at 60× magnification showing A, microglia in the hippocampus identified by anti-IBA1 contiguous cytoplasmic staining and B, mast cells in the thalamus identified by toluidine blue staining and characteristic morphology with the presence of large metachromatic secretory granules filling the cytoplasm and a unilobular ovoid nucleus (denoted by arrows).

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Table 1. Fetal plasma cytokine measurements (pg/mL).

|       | IL-1β |       | IL-6  |
|-------|-------|-------|-------|
|       | Day 1 | Day 4 |       |
|       | pre UCO 1 | post UCO 6 | pre UCO 1 | post UCO 6 |
| Controls (8) | 508±95 | 477±90 | 423±102 | 526±94 |
| Mild UCO (6) | 640±126 | 672±153 | 527±70 | 509±95 |
| Mod UCO (5) | 645±119 | 657±196 | 755±158 | 874±173 |
| Severe UCO (7) | 1017±264 | 1032±219 | 846±258 | 1135±309 |
| Controls (6) | 336±152 | 328±149 | 321±150 | 321±150 |
| Mild UCO (5) | 271±178 | 253±165 | 264±171 | 252±174 |
| Mod UCO (4) | 353±239 | 278±181 | 309±174 | 318±185 |
| Severe UCO (7) | 478±169 | 501±203 | 394±168 | 335±135 |

Data are presented as mean ± SEM. Animal numbers for each of the groups are shown in parentheses.

Fetal Plasma Cytokine Measurements

Plasma cytokine measurements at the selected time points from fetal arterial blood sampling are shown in Table 1. Fetal IL-1β values showed considerable variance across the animal groups, being lowest in the control animals and highest in the severe UCO animals. However, for each of the animal groups there was no significant change in these values over the four days of study and thereby in relation to intermittent UCO insults. Fetal IL-6 values were also variable across the animal groups and again showed no significant change over the four days of study and thereby in relation to the UCO insults. Maternal IL-1β and IL-6 values also showed no significant change from that prior to the first UCO on day 1, and overall averaged 450±66 and 365±85 pg/mL, respectively, for all of the animal groups. However, maternal IL-1β and IL-6 values were again highest in the severe UCO animals at 522±97 and 572±176 pg/mL, respectively.

Baseline cytokine findings for individual animals as measured prior to the first UCO on day 1 were analyzed for significant correlations to further assess the relationship of fetal to maternal values, and of IL-1β to IL-6 values. Fetal IL-1β and IL-6 values at baseline were found to be well correlated with corresponding maternal IL-1β and IL-6 values, r = 0.75 and 0.65, respectively (both p<0.001). Likewise, fetal and maternal IL-1β values at baseline showed a modest correlation with corresponding IL-6 values, r = 0.44 (p<0.05) and 0.62 (p<0.01), respectively. Moreover, there was a gender effect with female fetuses showing higher IL-1β levels than males, 858±117 versus 481±101 pg/mL (p<0.05) as did their mothers, 432±83 versus 324±89 pg/mL, although this was not statistically significant. This accounted in part for the higher fetal IL-1β values in the severe UCO animals which had a disproportionate number of female fetuses at 5 out of 7 in comparison to the other animal groupings, at 50% or less.

The two animals with fetal pHa falling to 7.23 on the last day of study both showed an apparent systemic inflammatory response when compared to baseline control values with one showing a ~3 fold increase in IL-1β levels to 2618 pg/mL, while the other showed an ~8 fold increase in IL-6 levels to 1026 pg/mL.

Histochemical Scoring for Microglia and Mast Cells

Microglia immunoreactivity was analyzed at the end of the four days of intermittent UCO as a measure of local inflammatory response within the brain. In the control group animals regional differences were evident with the microglia cell counts in the white matter at 47.6±7.7 cells/HPF higher than that in all other regions (p<0.05) (Figure 3). For the UCO groups of animals the microglia analyzed separately. The remaining animals showed no overall change in their 5 minute pre UCO blood gas and pH values, nor in the blood/pHa response with each UCO as measured on days one and four of study. Accordingly, these were averaged for the respective groups as shown in Figure 2. As such, intermittent UCOs as studied for the mild, moderate, and severe UCO groups resulted in a transitory decrease in fetal PaO2 by ~10, 13, and 16 mmHg; in fetal pHa by ~0.04, 0.09, and 0.14; and a transitory increase in fetal PaCO2 by ~5, 13, and 20 mmHg, respectively (all p<0.01), but with a rapid return toward pre-occlusion values as measured 5 minutes after release of the occluder cuff (Figure 2). The three animals with worsening acidemia on day four of study with pHa values at 7.23, 7.23, and 7.28 as measured 5 minutes after the last UCO, were additionally moderately hypoxic this day with PaO2 values at 12.0, 16.3, and 16.9 mmHg, respectively, prior to the onset of UCOs.
cell counts were likewise highest in the white matter, but there were no significant differences from respective control values for any of the brain regions studied (Figure 3).

Mast cell distribution was analyzed at the end of the four days of study as a second measure of the local inflammatory response within the brain. In the control group animals regional differences were again evident with the mast cell counts in the choroid plexus while extremely low at 0.6 ± 0.2 cells/HPF, still higher than that in all other regions (p < 0.05) (Figure 4). For the UCO groups of animals the mast cell counts were likewise highest in the choroid plexus, but there were no significant differences from respective control values for any of the brain regions studied (Figure 4).

None of the three UCO animals with worsening acidemia on day four of study showed notable microglia cell changes, although the two animals with fetal pHs falling to 7.23 did show high mast cell counts in the choroid plexus at 2.7 and 1.9 cells/HPF.

**Discussion**

Intermittent UCOs as studied resulted in transitory fetal hypoxemia with associated acidemia which was most pronounced in the severe UCO group as expected with umbilical blood flow occluded the longest. The UCO induced acidemia can be attributed to the fetal hypercapnia also noted, as well as related increases in lactate levels likely indicating the onset of anaerobic metabolism by some fetal tissues including carcass and muscle with the redistribution of blood flow away from these tissues as we have previously shown [4]. However, while UCO insults resulted in moderate to severe degrees of fetal hypoxia and hypercapnia, for the most part there was complete recovery post occlusion with no cumulative blood gas or pH changes observed throughout the 4 days of study either at baseline pre-occlusion or during the UCOs. This would indicate that intermittent UCO up to 3 minutes in
duration are unlikely to have a residual impact on umbilical blood flow or cotyledonary blood gas exchange. Exceptions were the two moderate and one severe UCO group animals with moderate hypoxemia and worsening acidemia on day four of study and indicating some degree of altered uteroplacental gas exchange, whether related to the intermittent UCOs or otherwise.

Fetal arterial IL-β and IL-6 values showed no significant change as measured pre-term over the four days of intermittent UCO
insults regardless of their severity. This is in contrast to studies in humans where birth asphyxia with concerning hypoxic-acidemia has been shown to result in elevated cytokines in umbilical cord blood including IL-6 [17,18], and to our study in near term fetal sheep where repetitive UCO leading to severe acidemia resulted in a 2 fold increase in plasma IL-1β values [1]. As such, intermittent UCO in the absence of worsening acidemia is not a sufficient stimulus to evoke a systemic inflammatory response with increases in IL-1β and IL-6 plasma values. This is consistent with the short half-life of cytokines and need for repeated stimuli for continued production [8]. It is thus of interest that the two animals with sustained acidemia and pH falling to 7.23 on the last day of study both showed a systemic inflammatory response with increases in IL-1β and IL-6 plasma levels, and indicating that sustained hypoxia with worsening acidemia are sufficient stimuli for such response.

Fetal IL-1β and IL-6 values at baseline for individual animals were well correlated with corresponding maternal values. This is to be expected if the placenta is a primary source for these cytokines and with related secretion into both circulations, since IL-1β and IL-6 are unlikely to cross the cotyledonary placenta given their molecular weight [29]. Accordingly, the variance in IL-1β and IL-6 plasma values across the animals may relate in part to the initial surgical preparation and recovery from such, and the extent to which utero-cotyledonary tissues were traumatized with resultant inflammation leading to differing secretion rates for these cytokines. Fetal and maternal IL-1β values at baseline also showed a modest correlation with corresponding IL-6 values, which is again to be expected since IL-1β is known to stimulate expression of IL-6 in various cell types including leukocytes and endothelium through autocrine, paracrine and/or endocrine mechanisms [8,30]. Moreover, this observed relationship might also reflect the joint involvement of these two cytokines in the utero-cotyledonary inflammatory response to surgery and related secretion into both circulations. Of additional interest, female fetuses were found to have higher IL-1β levels than males, indicating that gender may impact on the fetal inflammatory response to various stimuli, in this case presumably the surgical manipulation several days earlier. This is consistent with the finding in patients delivering preterm whereby those with female infants are more likely to show placental inflammation [31] and have higher amniotic fluid IL-1 receptor antagonist levels as a homeostatic counter measure [22], but to our knowledge there has been little other study of fetal gender and immune responses. Nonetheless, there has been considerable human and animal-based study in adults of gender effects on immune responses. For the most part these support the contention of enhanced immune responses in females compared to males which is likely hormonally mediated [23,24], although this may well depend upon the immune response trigger and be different for infectious versus aseptic inflammatory stimuli [23–25].

Microglia and mast cell counts were found to be highest in the white matter and choroid plexus, respectively, as we have previously reported for the ovine fetus near term [1], but there was no evident impact of the intermittent UCO insults on these cell counts for any of the brain regions studied. This again is in contrast to our study in near term fetal sheep with repetitive UCO leading to severe acidemia which resulted in a ~2 fold increase in microglia cell counts in the white matter and hippocampus, and a ~2 fold increase in mast cell counts in the choroid plexus and de novo appearance in the thalamus [1]. As such, intermittent UCOs as studied and known to result in cerebral ischemia-reperfusion [4,5], but in the absence of worsening acidemia and evident systemic inflammation, are not a sufficient stimulus to evoke a local inflammatory response within the brain with increases in microglia and mast cell counts. This would support our contention that an increase in circulating cytokines is required to modulate the brain inflammatory response given their ability to increase blood-brain barrier permeability to macrophages and other cellular and molecular inflammatory mediators [8,19]. In this regard, the two animals with worsening acidemia on the last day of study and apparent systemic inflammation also showed high mast cell counts in the choroid plexus as further support for the role of circulating cytokines in the brain inflammatory response.

We have previously shown that intermittent UCOs of 90 second duration in both preterm and near term fetal sheep can lead to a low level of necrotic appearing cells in the gray matter [14] and a marginal increase in apoptotic appearing cells in the hippocampus [15]. This could involve inflammatory mediators which can become injurious by activating apoptotic and necrotic pathways in the developing brain [8,32] and might be increased systemically in response to placental hypoxia and/or hypoperfusion with cord compression [2,3], and locally within the brain in response to ischemia-reperfusion with an increase in reactive oxygen species [4–7]. We have now determined that intermittent UCOs of up to 3 minutes duration with severe, but limited fetal hypoxemia and no cumulative blood gas or pH changes as studied over a 4 day period, do not result in either a systemic or local inflammatory response within the brain. To the extent that intermittent cord compression does occur over the latter part of human pregnancy [9–11] and relates to adverse neurodevelopment [12,13], the present findings would indicate that this is unlikely to involve inflammatory pathway mechanisms. We have also determined that the cotyledonary placenta in sheep is likely to be a primary source of inflammatory cytokines with related secretion into both the maternal and fetal circulations. Moreover, this linkage between maternal and fetal immune responses appears to be impacted by fetal gender, being heightened in females and warrants further study.

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Author Contributions

Conceived and designed the experiments: MGF BM BSR. Performed the experiments: APP MGF BM BSR. Analyzed the data: APP MGF RV RH BM BSR. Contributed reagents/materials/analysis tools: MGF RV RH BM BSR. Wrote the paper: APP MGF BSR. Provided reagents, technical support for the cytokine analysis: RV. Provided technical support for the mast cell and microglia analysis: RH.

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