Reprod. Fertil. Dev., 1996, **8**, 317–21

Relation between Cerebral Oxygen Delivery and Neuronal Cell Damage in Fetal Sheep near Term

Richard Berger[AC], Tina Lehmann[A], Jochen Karcher[A], Walter Schachenmayr[B] and Arne Jensen

[A] *Department of Obstetrics and Gynaecology, Justus-Liebig-Universität Gießen, Klinikstr. 32, D-35385 Gießen, Germany.*
[B] *Department of Neuropathology, Justus-Liebig-Universität Gießen, Arndtstr. 16, D-35385 Gießen, Germany.*
[C] *Present address and address for correspondence: Universitätsfrauenklinik Bochum, Knappschaftskrankenhaus, In der Schornau 23–25, 44892 Bochum, Germany.*

Abstract. Asphyxia is one of the major causes for fetal brain damage. Although the quality of life of the so affected children is mostly very limited, the pathogenesis of hypoxic fetal brain damage is poorly understood. Particularly, there is a lack of studies, in which cerebral oxygen delivery is directly correlated to the extent of neuronal cell damage in the same brain specimens. Therefore, we measured cerebral oxygen delivery before (−1 h), during (+3 min & +27 min) and after (+10 min, +4 h, +72 h) 30 min of ischaemia in 5 chronically catheterized normoxemic fetal sheep at 129±1 days gestation (term is at 147 days) using the microsphere method. In contrast to previous studies (Williams *et al.* 1990), we arrested carotid arterial blood flow above the lingual artery for 30 min during surgery. Seventy-two hours later the fetal brains were fixed *in vivo* under barbiturate anaesthesia of both the fetus and the ewe. After cerebral blood flow analysis neuronal cell damage was assessed with light microscopy in 43 specimens of the fetal brain after cresyl violet/fuchsin staining using a scoring system. After arrest of carotid arterial blood flow cerebral blood flow was reduced by 80%. Neuronal cell damage was focussed on the cerebral cortex. Almost no damage could be detected in deeper parts of the brain. In the cerebrum there was a threshold oxygen delivery of 3 ml O_2/100 g tissue/min, below which neuronal damage occurred. However, there was no correlation between cerebral oxygen delivery and neuronal cell damage in specimens of the cerebrum, in which oxygen delivery was less than 3 ml O_2/100 g tissue/min, suggesting selective vulnerability. Therefore, in addition to the reduction in cerebral oxygen delivery, other variables, e.g. neurotransmitter release, receptor pattern or oxygen radicals, may be involved in the development of brain damage.

Introduction

Hypoxic–ischaemic injury to the fetal and neonatal brain represents one of the major causes of neurodevelopmental morbidity in the term infant (Volpe, 1987). Although the quality of life of the affected children is mostly very limited (Low *et al.* 1985; Ellenberg and Nelson 1988; Freeman and Nelson 1988), the pathogenesis of hypoxic fetal brain damage is poorly understood (Myers 1977; Lou 1988). In particular, the interrelation between the severity of the insult and the amount of cerebral neuronal cell damage has not been determined. Therefore, we designed a study in chronically catheterized fetal sheep near term, in which we arrested carotid arterial blood flow acutely, i.e. during surgery, to correlate cerebral oxygen delivery with the extent of neuronal cell damage.

Material and Methods

Animal Preparation

Five fetal sheep were chronically prepared at a gestational age of 129±1 days (term is at 147 days). All ewes were anaesthetized by subarachnoid injection of 8 ml of 0·75% (w/v) bupivacaine at the lower spine, and were operated on under sterile conditions. Polyvinyl catheters were placed in a maternal iliac artery and vein through tibial vessels. The abdominal wall was opened in the midline and, through a small uterine incision, the fetal hindlimbs were exposed. Under local anaesthesia with 1·0% (w/v) prilocaine HCl, polyvinyl catheters were inserted via the pedal vein of each hindlimb into the inferior vena cava. The uterine incision was closed and a second uterine incision was made over the fetal head. The head and neck of the fetus were exteriorized. To prevent the fetus from breathing, the head was covered by a water-filled rubber glove. Catheters were inserted into both fetal brachial arteries. After control measurements of the physiological variables and of cerebral blood flow, both fetal common carotid arteries were prepared. We arrested cerebral blood flow during the course of surgery using a modified method originally described by Williams *et al.* (1990). Both carotid arteries were occluded above the lingual arteries for 30 min by kinking the vessels using small plastic rods, while blood flow measurements were performed as described below. At the end of the cerebral ischaemic period a catheter was placed into the amniotic cavity, and the second intrauterine incision was closed. All catheters were filled with heparin (1000 IU/ml), plugged, and passed subcutaneously to the ewe's flank, where they were exteriorized and protected by a pouch sewn to the skin. On the day of surgery and each day thereafter, the ewe received 2 million units of penicillin

1031-3613/96/030317$05.00

G (Grünenthal, Germany) and 80 mg of gentamycin sulfate (Merck, Germany), half intravenously and half into the amniotic cavity.

Experimental Protocol

To determine the time course of fetal cerebral blood flow before (−1 h), during (+3 min and +27 min), and after (+10 min, +4 h and +72 h) 30 min of cerebral ischaemia, six batches of differently isotope-labelled microspheres (^{113}Sn, ^{103}Ru, ^{46}Sc, ^{114}In, ^{141}Ce and ^{95}Nb, 15·5 μm diameter; New England Nuclear) were injected under sterile conditions into the inferior vena cava using a mixing chamber, while reference samples from the brachial artery were withdrawn at a rate of 2·5 ml/min for 90 s (Rudolph and Heymann, 1967). Before injection the microspheres were sonicated and checked for size, shape, and aggregation. Since there were no differences in cerebral blood flow between the two hemispheres, mixing of the microspheres during the injection procedure was sufficient. The amount of injected microspheres was large enough (about 1 million) to ensure both an adequate number of microspheres per sample and valid blood flow measurements during cerebral ischaemia (Buckberg *et al.* 1971; Jensen *et al.* 1987*a*, 1987*b*). Specific calculations revealed that about 400 microspheres were trapped in low-flow cerebral areas during ischaemia. Thus, for theoretical considerations the blood-flow estimates in these areas are within 5% of the true values (Buckberg *et al.* 1971). During the injection of microspheres, fetal heart rate and the ascending aortic and intrauterine pressures were recorded simultaneously. At the fifth injection of microspheres (+4 h) no pressure measurements were performed for technical reasons. Before each injection blood samples were obtained from the brachial artery to measure blood gases, oxygen saturation of haemoglobin, and acid–base balance. At the end of the experiment (+72 h) the ewe was given a lethal dose of sodium pentobarbitone and saturated potassium chloride intravenously, and the fetus was perfused with 300 ml formalin (15%, w/v, saline).

The experimental protocols were approved by the appropriate institutional review committee and meet the guidelines of the responsible governmental agency.

Measurements

Ascending aortic and intrauterine pressure and fetal heart rate were recorded on a polygraph (Hellige, Germany). Blood gases and pH were measured in an automatic blood gas analyser (278 Blood Gas System; Ciba Corning, Frankfurt, Germany), and base excess was calculated. Haemoglobin concentration and oxygen saturation of haemoglobin were measured photometrically (OSM 2 Hemoximeter; Radiometer, Copenhagen, Denmark) in duplicate. Oxygen content of the blood was calculated according to the formula: [oxygen content (ml/dl) = haemoglobin (g/dl)×1·34×oxygen saturation of haemoglobin (%)/100].

Fetal cerebral blood flow and the extent of neuronal cell damage were assessed in the same brain specimens. To determine fetal cerebral blood flow the fetal brain was removed and fixed in formalin for at least seven days. Afterwards, the cerebrum was separated from the basal ganglia and divided into four frontal sections with a thickness of about 1·5 cm. The right and left parts of these frontal sections were further subdivided into four equally sized segments each weighing 1–2 g. In addition to these 32 specimens from the cerebrum, specimens from caudate nucleus, thalamus, hippocampus, tegmentum-colliculi-pons, cerebellum and spinal medulla were separated. These were placed into vials, which were filled to the same height to reduce variations in geometry. The solid-state semi-conductor germanium (Ge) gamma counter used had a high-energy resolution of about 3 keV and was connected to a multichannel (2048) pulse-height analyser (ND 62; Nuclear Data Inc., IL, USA). The results were normalized with respect to time and sample weight.

After calculation of cerebral blood flow the specimens of the brain were embedded in paraffin, coronally subserially sectioned to 10 μm and then stained with cresyl violet–fuchsin. Every 40th section was mounted to evaluate the extent of neuronal cell damage. Each section was assessed under a magnification of 250×. Neurons with ischaemic cell damage were identified according to the criteria of Brown and Brierley (1971). Neuronal cell damage in each microscopical visual field was quantified according to the proportion of neurons that were dead: 0–5% (score 1), 5–50% (score 2), 50–95% (score 3), 95–99% (score 4), 100% (score 5). All three observers (T.L., J.K., R.B.) involved in the histological evaluations were blind to the cerebral blood flow measurements. The intra-observer reliability was assessed as the coefficient of variation when each observer analysed the histological slices of one brain specimen ten times; it amounted to 2% (T.L.), 4% (J.K.) and 4% (R.B.). The inter-observer reliability was assessed by all observers analysing the histological slices of a selection of ten different brain specimens; the results were compared and yielded a 10% coefficient of variation between observers.

To exclude brain damage due to the preparation itself, we measured cerebral blood flow and neuronal cell damage in two sham control fetuses using the same protocol without arresting carotid arterial blood flow. There was no change in cerebral blood flow during the 72-h period (control value: 249·5 ml/100 g/min). Furthermore, no cerebral cell damage could be detected in any specimens of the brain examined.

Calculations

Fetal cerebral blood flow was calculated from counts of the injected nuclide recovered in the fetal cerebrum and the appropriate reference sample, and from the withdrawal rate of the reference sample (Rudolph and Heymann, 1967). Cerebral oxygen delivery was calculated by the product of cerebral blood flow and oxygen content determined in the brachial artery and expressed as ml O_2/100 g tissue/min.

The histological score of each cerebral specimen was calculated by averaging the scores of all visual fields from three sections of that specimen. The number of scored visual fields per specimen ranged between 400 and 500.

Statistical Analysis

Results are given as means ± s.e.m. The physiological variables and blood flow measurements were analysed statistically using a single-factor once-repeated-measure ANOVA model. Differences in neuronal cell damage and oxygen delivery between various sections of the cerebrum were tested by a three-factor non-repeated-measures ANOVA model. As *post-hoc* testing procedure the Fisher test was used (statistical package Stat View; Abacus Concepts Inc., Berkerly, CA, USA).

Results

In the control period, blood gases, pH, and heart rate were within the normal range for chronically prepared fetal sheep (Jensen and Berger, 1991), whereas blood pressure, cerebral blood flow and plasma concentration of both glucose and lactate were slightly increased (Tables 1 and 2). After carotid arterial blood flow had been arrested for 30 min, cerebral blood was reduced by 80% with a considerable inter-individual variation. Cerebral blood flow during ischaemia was lower in the cerebrum than in the deeper parts of the brain. In the immediate recovery period (+10 min) there was a variable cerebral hyper-

Table 1. Acid–base balance, blood gases, plasma concentration of glucose and lactate, fetal heart rate (FHR), and blood pressure (BP) in fetal sheep before, during and after 30 min of cerebral ischaemia

Values are means ± s.e.m. Significant versus control within groups: [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$

	Control	Ischaemia		Recovery		
		+3 min	+27 min	+10 min	+4 h	+72 h
pH	7·36±0·02	7·37±0·02	7·37±0·02	7·35±0·02	7·37±0·02	7·39±0·01
pO_2 (mmHg)	18·4±1·7	20·2±1·0	19·7±1·3	17·0±0·8	20·9±1·4	22·9±3·7
pCO_2 (mmHg)	48·2±1·4	46·0±1·1	46·1±0·9	46·8±1·0	52·1±0·5[b]	47·9±1·0
BE[A]	1·9±1·1	1·7±1·0	1·5±1·0	0·7±0·9	4·1±1·7[a]	4·7±0·8[b]
SO_2 (%)[B]	53·4±2·1	60·9±2·9	58·1±3·0	50·0±4·5	62·0±2·7	59·3±5·3
Glucose (mg/ml)	81·75±6·8	75·8±5·6	73·6±7·9	69·8±7·8	30·2±5·7[c]	25·0±1·2[c]
Lactate (mmol/l)	3·73±0·17	3·93±0·40	4·43±0·51	4·95±0·49[a]	3·6±0·41	1·88±0·36[b]
FHR (min^{-1})	175±12	162±17	175±13	191±20	—	147±4
BP (mmHg)	62±5	69±6	71±6	66±5	—	55±4

[A] BE, base excess. [B] SO_2, oxygen saturation.

Table 2. Total and regional cerebral blood flow (ml/100 g tissue/min) in fetal sheep before, during and after 30 min of cerebral ischaemia

Values are means ± s.e.m. Significantly different from control values: [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$

	Control	Ischaemia		Recovery		
		+3 min	+27 min	+10 min	+4 h	+72 h
Cerebrum	214·2±42·2	41·5±21·6[a]	47·1±11·5[a]	301·4±116·1	122·3±22·4	248·6±51·2
Caudate nucleus	281·3±72·4	40·7±21·7[a]	63·4±14·9[a]	329·2±126·4	163·8±31·2	301·8±57·6
Thalamus	377·9±78·8	74·9±39·3[b]	75·7±15·7[b]	287·6±57·5	207·8±38·9	358·1±88·8
Hippocampus	220·9±53·1	68·9±44·7[a]	51·5±13·3[a]	216·6±70·4	125·1±27·9	223·3±52·2
Colliculi, tegmentum, pons	416·2±84·1	83·7±39·4[b]	83·9±18·9[b]	271·7±23·5	240·6±46·8	443·1±106·8
Cerebellum	309·1±53·8	59·1±23·7	76·2±15·9	508·0±200·7	204·7±35·7	380·2±96·8
Medulla	446·1±84·4	117·7±45·1[c]	109·6±23·1[c]	331·2±37·6	235·9±35·8[a]	454·1±116·1
Total brain	254·4±50·5	51·2±26·0[a]	55·9±13·3[a]	302·4±99·5	144·4±25·8	283·0±61·3

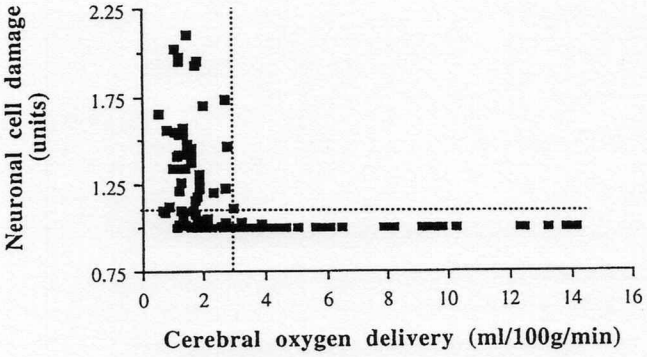

Fig. 1. Relation between cerebral oxygen delivery (ml O_2/100 g tissue/min) and neuronal cell damage (units). If cerebral oxygen delivery falls below 3 ml O_2/100 g tissue/min, neuronal cell damage occurs (χ^2 70·66; $P < 0.001$).

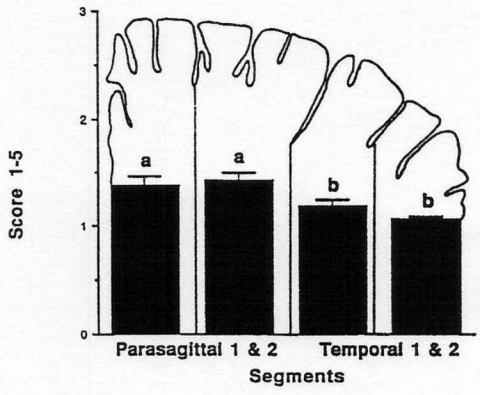

Fig. 2. Mean scores of neuronal damage of those cerebral segments in which a reduction of oxygen delivery below 3 ml O_2/100 g tissue/min was achieved. Values are means ± s.e.m. Differences between columns marked with 'a' and 'b' are significant ($P < 0.05$).

perfusion, followed by a hypoperfusion (+4 h). Finally, 72 h after cerebral ischaemia, blood flow to the brain recovered to control values (Table 2). No considerable changes in blood gases, pH, and heart rate could be detected, whereas the initial increase in arterial blood pressure and the increase in the plasma concentration of both glucose and lactate normalized at the end of the experiments (Table 1).

Neuronal cell damage caused by cerebral ischaemia selectively occurred in the cerebral cortex. However, the extent ranged between a maximum score of 2·10 in some parasagittal areas to a minimum score of 1·0 (no damage) in temporal areas (Figs 1 and 2). The damage was more severe in the depths of the sulci than in the gyri. Except for one fetus, in which a moderate neuronal cell damage in the hippocampus was observed (score 1·15), there was no damage at all in deeper parts of the brain (score 1·0±0·0).

In Fig. 1 the relation between cerebral oxygen delivery during cerebral ischaemia and neuronal cell damage is

shown for 160 specimens of the cerebrum derived from five fetal sheep. Cerebral oxygen delivery during cerebral ischaemia was calculated as the average of cerebral oxygen delivery at 3 min and 27 min after carotid arterial blood flow had been arrested. Interestingly, there was a threshold oxygen delivery of 3 ml O_2/100 g tissue/min, below which neuronal cell damage occurred. No side or section differences in neuronal cell damage were observed in those specimens of the cerebrum in which a reduction of oxygen delivery to less than 3 ml O_2/100 g/min was achieved. However, parasagittal segments within each hemisphere were more damaged than temporal segments, even though cerebral oxygen delivery during ischaemia was not different between segments (Fig. 2). Furthermore, no neuronal cell damage was observed in parts of the brain other than the cerebrum, even though oxygen delivery was below 3 ml O_2/100 g/min, except for the hippocampus of one fetus as described above. Finally, we were unable to detect any correlation between cerebral oxygen delivery before or after ischaemia and neuronal cell damage.

Discussion

The present study in fetal sheep near term demonstrates a clear-cut threshold of cerebral oxygen delivery of about 3 ml O_2/100 g tissue/min, below which neuronal cell damage in the cerebrum occurred after 30 min of cerebral ischaemia (Fig. 1). This threshold of cerebral oxygen delivery is in the same order of magnitude as that shown for adult animals (for review Astrup, 1982; for review Heiss, 1983). However, neuronal cell damage did not always occur in the cerebrum, even though oxygen delivery was less than 3 ml O_2/100 g tissue/min (Fig. 1), suggesting selective vulnerability (Brierley and Brown 1982; Brierley and Graham 1984; Pulsinelli 1985; Schmidt-Kastner *et al.* 1990). This view is supported by the fact that, first, neuronal cell damage happened to be focussed on parasagittal cerebral areas independent of oxygen delivey (Fig. 2). Secondly, almost no neuronal cell damage was observed in parts of the brain other than the cerebrum in spite of an oxygen delivery below 3 ml O_2/100 g tissue/min. Therefore, in addition to the reduction in cerebral oxygen delivery, other variables, e.g. neurotransmitter release, receptor pattern and/or oxygen radicals, may be involved in the development of brain damage (Siesjö and Bengtsson 1988; for review Krieglstein and Oberpichler 1992). The observed inter-animal variability in neuronal cell damage may be related to the variation in reduction of cerebral blood flow during ischaemia.

The observed increases in arterial blood pressure, cerebral blood flow and plasma concentrations of both glucose and lactate during the control period were due to the acute experimental procedure to produce cerebral ischaemia, rather than to a preexisting disturbance of placental gas exchange. This is supported by the fact that after 72 h, when the fetuses had recovered from surgery, blood pressure and plasma concentrations of both glucose and lactate returned to normal values (Jensen and Berger 1991). It is unlikely that elevated blood glucose concentrations, as observed in this study, have aggravated the extent of neuronal damage, because this has been described only at excessively high glucose concentrations (Myers and Yamaguchi 1976; Kalimo *et al.* 1981; Rehncrona *et al.* 1981). In spite of a few disadvantages of our acute cerebral ischaemia model, the clear advantage is that total arrest of carotid arterial blood flow can be ensured and controlled visibly. Thus, cuff failures (Williams *et al.* 1990) and consequent wastage of animals can be avoided.

References

Astrup, J. (1982). Energy-requiring cell functions in the ischaemic brain. *J. Neurosurg.* **56**, 482–97.

Brierley, J. B., and Brown, A. W. (1982). The origin of lipid phagocytes in the central nervous system: I. The intrinsic microglia. *J. Comp. Neurol.* **211**, 397–406.

Brierley, J. B., and Graham, D. I. (1984). Hypoxia and vascular disorders of the central nervous system. In 'Greenfield's Neuropathology'. (Eds J. H. Adams and J. A. N. Corsellis.) pp. 125–207. (Edward Arnold: London.)

Brown, A. W., and Brierley, J. B. (1971). Anoxic–ischaemic cell change in rat brain light microscopic and fine structural observations. *J. Neurol. Sci.* **16**, 59–84.

Buckberg, G. D., Luck, J. C., Payne, D. B., Hoffman, J. I. E., Archie, J. P., and Fixler, D. E. (1971). Some sources of error in measuring regional blood flow with radioactive microspheres. *J. Appl. Physiol.* **31**, 598–604.

Ellenberg, J. H., and Nelson, K. B. (1988). Cluster of perinatal events identifying infants at high risk for death or disability. *J. Pediatr.* **113**, 546–52.

Freeman, J. M., and Nelson, K. B. (1988). Intrapartum asphyxia and cerebral palsy. *Pediatrics* **88**, 240–9.

Heiss, W. D. (1983). Flow thresholds of functional and morphological damage of brain tissue. *Stroke* **14**, 329–31.

Jensen, A., and Berger, R. (1991). Fetal circulatory responses to oxygen lack. *J. Dev. Physiol.* **16**, 181–207.

Jensen, A., Hohmann, M., and Künzel, W. (1987*a*). Redistribution of fetal circulation during repeated asphyxia in sheep: effects on skin blood flow, transcutaneous PO_2, and plasma catecholamines. *J. Dev. Physiol.* **9**, 41–55.

Jensen, A., Hohmann, M., and Künzel, W. (1987*b*). Dynamic changes in organ blood flow and oxygen consumption during acute asphyxia. *J. Dev. Physiol.* **9**, 543–59.

Kalimo, H., Rehncrona, S., Söderfeldt, B., Olsson, Y., and Siesjö, B. K. (1981). Brain lactic acidosis and ischemic cell damage: 2. Histopathology. *J. Cereb. Blood Flow Metab.* **1**, 313–27.

Krieglstein, J., and Oberpichler, H. (1992). 'Pharmacology of Cerebral Ischemia.' (Wissenschaftliche Verlagsgesellschaft: Stuttgart.)

Lou, H. C. (1988). Perinatal hypoxic–ischemic brain damage and intraventricular hemorrhage. *Baillieres Clin. Obstet. Gynecol.* **2**, 213–20.

Low, J. A., Galbraith, R. S., Muir, D. W., Killen, H. L., Pater, E. A., and Karchmar, E. J. (1985). The relationship between perinatal hypoxia and newborn encephalopathy. *Am. J. Obstet. Gynecol.* **152**, 256–60.

Myers, R. E. (1977). Experimental models of perinatal brain damage: relevance to human pathology. In 'Intrauterine Asphyxia and the Developing Fetal Brain'. (Ed. L. Gluck.) pp. 37–97. (Year Book Publ. Co.: New York.)

Myers, R. E., and Yamaguchi, M. (1976). Effects of serum glucose concentration on brain response to circulatory arrest. *J. Neuropath. Exp. Neurol.* **35**, 301.

Pulsinelli, W. A. (1985). Selective neuronal vulnerability: morphological and molecular characteristics. *Prog. Brain Res.* **63**, 29–37.

Rehncrona, S., Rosen, I., and Siesjö, B. K. (1981). Brain lactic acidosis and ischemic cell damage: 1. Biochemistry and neurophysiology. *J. Cereb. Blood Flow Metab.* **1**, 297–311.

Rudolph, A. M., and Heymann, M. A. (1967). Circulation of the foetus *in utero*: methods for studying distribution of blood flow, cardiac output and organ blood flow. *Circ. Res.* **21**, 163–84.

Schmidt-Kastner, R., Szymas, J., and Hossmann, K.-A. (1990). Immunohistochemical study of gial reaction and serum-protein extravasation in relation to neuronal damage in rat hippocampus after ischemia. *Neuroscience* **38**, 527–40.

Siesjö, B. K., and Bengtsson, F. (1988). Calcium, calcium antagonists, and ischemic cell death in the brain. In 'Pharmacology of Cerebral Ischemia'. (Ed. J. Krieglstein.) pp. 23–9. (Wissenschaftliche Verlagsgesellschaft: Stuttgart.)

Volpe, J. J. (1987). 'Neurology of the Newborn.' (WB Saunders: Philadelphia.)

Williams, C. E., Gunn, A. J., Synek, B., and Gluckman, P. D. (1990). Delayed seizures occurring with hypoxic–ischemic encephalopathy in the fetal sheep. *Pediatr. Res.* **27**, 561–5.

Manuscript received by *J. Dev. Physiol.* 13 August 1993