

ORIGINAL ARTICLES

Posthypoxic cooling of neonatal rats provides protection against brain injury

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Abstract

Aim—To determine whether moderate hypothermia, applied after a hypoxic-ischaemic insult in neonatal rats, reduces cerebral damage.

Method—Unilateral hypoxic-ischaemic brain damage was induced in 7 day old rats by left carotid ligation, followed by 120 minutes of normothermic exposure to 8% O₂, followed by random selection to three hours of hypothermia (rectal temperature, mean (SD), 32.5 (0.4)°C) or normothermia (38.3 (0.4)°C). One hundred and one animals were used for brain temperature or blood chemistry studies and 24 for survival studies (7 days) with neuropathology, including cell counting as outcome measures.

Results—Thirty sections from each brain were histologically examined with respect to distribution and pattern of damage and given a score from 0 to 4. Animals treated with hypothermia had significantly less damage than normothermic animals (score 0.5 (0.3) vs 1.8 (0.5)).

Conclusions—Posthypoxic hypothermia reduces brain damage in awake, unrestrained 7 day old rats.

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Keywords: ischaemia, hypothermia, newborn, rat.

Despite advances in obstetrics, infants continue to be damaged by severe hypoxia-ischaemia during labour and delivery. After initial cardiorespiratory resuscitation, there are no clinical interventions that have improved the outcome of the encephalopathy.^{1,2} It is clear that destructive processes continue to damage the brain for many hours after oxygenation and circulation have been restored. These processes involve calcium entry, oxygen free radicals, excitatory amino acids, proteolytic activation and immune/inflammatory activation.

Pharmacological treatment applied after the insult has provided partial neuroprotection (evaluated as a quantitative reduction in brain lesion) in an immature animal model.³ However, such intervention has not yet been investigated in humans and may have serious side effects in newborns.⁴

Reducing the cerebral temperature during hypoxia-ischaemia significantly protects the brain against damage in both humans and experimental animals⁵ and is a standard procedure during cardiac and neurosurgery. However, the possibility of reducing cerebral injury by cooling after a cerebral insult has not been so fully researched. Although not based on controlled studies, it was suggested in the 1960s that infants cooled following birth asphyxia showed lower mortality and morbidity at 1 to 2 years of age.^{6,7} At the same time a study by Silverman suggested that premature infants had a higher mortality when nursed at 31.7°C incubator temperature for five days than at 33.3°C,⁸ and this significantly reduced the enthusiasm for cooling babies especially as no mechanistic framework then existed to explain any protective effect.

Hypothermia initiated after the insult has provided partial protection in some adult animal models,^{9,10} but not in others.^{11,12} In the immature rat subjected to three hours of mild or moderate hypothermia the treatment failed to show protection.¹³ However, in the newborn piglet, who has a more mature brain, 12 hours of mild posthypoxic-ischaemic hypothermia ameliorated delayed energy failure.¹⁴ We set out to examine the effect of three hours of moderate hypothermia in the immature rat, paying particular attention to stable temperature and unrestrained experimental conditions.

Methods

The investigation was approved by the Göteborg University animal research ethics committee. On postnatal day 7 (day of birth designated day 0) a total of 12 Sprague-Dawley rat pups from 18 unrestricted litters were either used in temperature studies (n=12), acute experiments for blood chemistry analysis (n=77), or survival experiments after a hypoxic-ischaemic insult (n=24); 39 were excluded. There were no differences in weight or sex ratio in any of the three subgroups studied. The animals were kept under standard conditions.¹⁵

All pups except six unligated controls were anaesthetised with enflurane (3.0–3.5% for induction, 0.5–1.0% for maintenance) and oxygen:nitrous oxide 1:1. The left common

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carotid artery was ligated and cut between two ligatures. The duration of this operation varied between 4.5 and 7 minutes. Animals were excluded from the study if they weighed less than 9 g ($n=1$) or there was bleeding during ligation ($n=2$). Two animals ('probe animals') in each of the 18 litters held rectal probes (1 mm in outer diameter, inserted 0.75 cm and taped on to the tail) to monitor the temperature during the whole experimental period and they were later excluded.

Postoperatively, the pups were returned to the dam, and two hours after completion of the last operation the animals were subjected to hypoxia (8% O₂ in N₂) in a double walled chamber (volume 4.2 litres). The air temperature was changed by a temperature regulated water jacket preset to give an air temperature of 36°C during the two hour long insult. All animals from the same litter were exposed simultaneously and in the same jar, except those used as controls who were not exposed to hypoxia. Immediately before exposure individual animals were paired for weight and within each pair randomly selected to be treated with either normal temperature (rectal temperature 38.5°C) or hypothermia (rectal temperature 32.5°C) for three hours directly following the insult. The probe animals from each litter (one to each temperature) were restrained in their movements so that the probe leads would not be entangled. The rest of the animals were allowed to move freely.

After hypoxia, half the litter was transferred to an identical pre-cooled jar (initial air temperature 15°C) for induction of hypothermia. This reduced rectal temperature (mean (SD) to 32.5 (0.7)°C within 18.4 (6.5) minutes and this remained stable throughout. During steady hypothermia the air temperature was 29.1 (1.2)°C. The other half of the litter stayed in the original jar at normothermia where the hypoxic gas was changed back to air. After three hours of hypothermia or normal temperature, after a total period of separation of 5.5 hours, the pups were returned to the dam.

Twenty four animals survived to day 14 when they were anaesthetised with pentobarbital intraperitoneally 1–2 mg and the brains were perfusion fixed (transcardiac) with 4% phosphate buffered (0.1 M) formaldehyde for 15 minutes. The brains were left in situ at

4°C for 24 hours before they were dissected out and kept in 4% formaldehyde until further processing. In 12 animals we recorded simultaneous brain and rectal temperatures. Under enflurane/N₂O anaesthesia, temperature sensors (type K, chromel-alumel; 0.2 mm diameter) were inserted 2 mm into each hemisphere (aiming at the caudo-putamen). The sensors were fixed by tape on to an external frame. The positions of the sensors were confirmed at necropsy. A rectal sensor was inserted as described. The animals were taped on to a grid restricting neck and leg movements and allowed to stabilise for 60 minutes inside the jar before the start of hypoxia followed by hypothermia. Continuous recordings of rectal and bilateral hemispheric temperatures were stored (Lab-View, version 2.5.2 National Instruments, Austin, Texas, USA). The temperature sensors were calibrated against a precision mercury thermometer before and after every experiment. There was little (<0.05°C) deviation in the values obtained.

Seventy seven animals were used to study blood chemistry changes at different points in time during the experiment when they were decapitated. Mixed arterial and venous blood samples from the neck were immediately obtained in duplicate. Blood gases were analysed (Radiometer ABL 510, Copenhagen, Denmark) in 13 controls (ligated $n=7$, unligated $n=6$) in eight animals after two hours of hypoxia, in eight after three hours at normal temperature, and in eight after three hours of hypothermia. Samples were analysed at body temperature. Glucose and lactate were examined in 10 control animals (ligated but no hypoxia) who were separated from the dam for five hours, and in 30 animals after the standardised insult followed by three hours of hypothermia ($n=15$) or normal temperature ($n=15$). Glucose and lactate were analysed by standard assays without deproteinisation (Hexokinase G6P-DH, Boehringer, Mannheim, Germany) and ultraviolet methods.

Coronal blocks through the brain were embedded in paraffin wax, subserially sectioned at 5 µm and stained with haematoxylin and eosin. Thirty sections were examined from each brain. In five sections around the mid dorsal level, total cell counting in the hippocampus (area CA¹⁻⁴ including gyrus dentatus) was also performed. The two examiners (EML and FA) were blinded to the mode of treatment.

The localisation of the brain damage obtained in the present study was the same as that found by Towfighi *et al.*¹⁶ who examined their rat pups 21 days after the insult. The pattern of damage followed predominantly the distribution of the middle cerebral artery. Typically, the lateral part of the hemisphere was the first to be affected. The predominant lesion in the present model was infarction, which after seven days of survival, before gliosis had developed, was seen as pale areas with vacuolisation of the neuropil and necrotic neurons with eosinophilic cytoplasm. The extent of this type of damage was graded from 0–4, with 0.5 intervals for each of the four

Table 1 Definition of neuropathology grading

Brain area	Grading	Percentage area affected	Morphological changes
Cortex, thalamus, and basal ganglia	1	≤10	Small, patchy, complete or incomplete infarcts
	2	20–30	Partly confluent, complete, or incomplete infarcts
	3	40–60	Large confluent complete infarcts
	4	>75	In cortex total disintegration of the tissue; in thalamus and basal ganglia large complete infarcts
Hippocampus*	1	≤20	Necrotic neurons only in the most lateral areas – CA ₂ and CA ₃
	2	50	Patchy areas of necrotic neurons in sectors CA ₁ –CA ₄
	3	75	More extensive areas of necrotic neurons in sectors CA ₁ –CA ₄
	4	100	Complete infarction of hippocampus including the dentate gyrus

Grade 0 indicates no histopathological damage.

*The grading system is defined differently for grades 1–3 in the hippocampus as selective neuronal necrosis is the typical damage in this area.

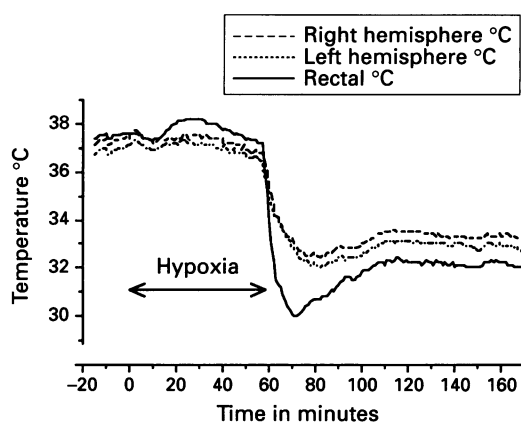


Figure 1 Simultaneous recordings of intracerebral temperatures from both hemispheres as well as rectal temperature in a 7 day old awake rat pup during 60 minutes of hypoxia (8% O₂) followed by 110 minutes of hyperthermia. Zero time is the start of hypoxia.

regions – the cortex, hippocampus, basal ganglia and thalamus. The pathological scores for these areas are defined in table 1. The term 'incomplete' infarct means a localised area where the neurons are mainly necrotic while the other tissue elements (such as glia and vessels) are preserved. In a completely infarcted area all tissue elements are damaged. The contralateral hemisphere was without damage in all animals.

Treatment groups were compared using the Wilcoxon matched pairs test. Blood chemistry values were compared using ANOVA followed by Student's *t* test with Bonferroni correction when appropriate. A *P* value of less than 0.05 was regarded as significant. Individual brain and rectal temperatures were compared using Pearson's correlation coefficient. Pearson's correlation coefficient was also used for comparing cell counting and pathology scores in the hippocampus.

Results

Figure 1 shows brain temperature from both hemispheres (the left carotid artery was ligated) and rectal temperature in one rat pup during 60 minutes of hypoxia followed by 110 minutes of hyperthermia. Figure 2A shows the correlation ($r=0.99$) between the right and left hemispheric temperature over a temperature range of 28–40°C during normoxia or hypoxia in 12 animals. In fig 2B, the correlation between the right hemispheric and rectal temperature in the same situations is displayed

Table 2 Blood gas analysis of mixed arterial and venous blood from four groups of 7 day old rats

Condition	Control* (n=13)	Hypoxia† (n=8)	Normal temp recovery‡ (n=8)	Hypothermic recovery¶ (n=8)
pH	7.43 (0.05)	7.31 (0.04) ^a	7.41 (0.02)	7.38 (0.02)
PCO ₂ (kPa)	6.5 (0.7)	2.7 (0.7) ^b	4.8 (0.5)	5.4 (0.5)
PO ₂ (kPa)	9.4 (1.7)	7.2 (2.5)	10.2 (0.7)	10.6 (1.9)
ABE (mmol/l)	6.7 (5.1)	-14.3 (5.5) ^b	1.3 (2.3)	1.3 (1.7)
Haematocrit	29 (2.2)	29.2 (1.7)	30.9 (1.1)	29.9 (1.2)

Values are mean (SD); hypoxia *vs* control ^a*P*<0.01, ^b*P*<0.001.

*Seven were ligated and six unligated, values were pooled because there was no difference between the two groups; †hypoxic animals after two hours of hypoxia; ‡normal temperature recovery after three hours of posthypoxic normothermia; ¶hypothermia recovery after three hours of posthypoxic hypothermia.

No animals had rectal probes.

showing a correlation of 0.96 between the two, hence rectal temperature is representative of brain temperature.

Blood gas values obtained before hypoxia (ligated and unligated animals), at the end of hypoxia and at the end of either normal temperature or hypothermia recovery are shown in table 2. The values reflect a considerable metabolic acidosis (base deficit -14.3 mmol/l) and hyperventilation at the end of the hypoxic period. There was no difference in pH, blood gas values, or haematocrit between the normal temperature and hypothermia animals at the end of the recovery period. Table 3 shows the glucose and lactate values for control animals which were separated from the dam for five hours and for animals treated with either normal temperature or hypothermia after hypoxia. There was a small but significant increase in glucose after hypothermia. In the surviving animals the body weight on day 14 was higher in hypothermic animals than normal temperature animals (24.4 (2.5) g and 22.5 (3.7) g, respectively; *P*<0.02).

The definitions used for scoring the histological damage in each brain area are listed in table 1. Figure 3 shows the hippocampal pathology score (0–4) for each animal in the normal temperature (*n*=12) and hypothermia groups (*n*=12). The protective effect of three hours of hypothermia was significant. The

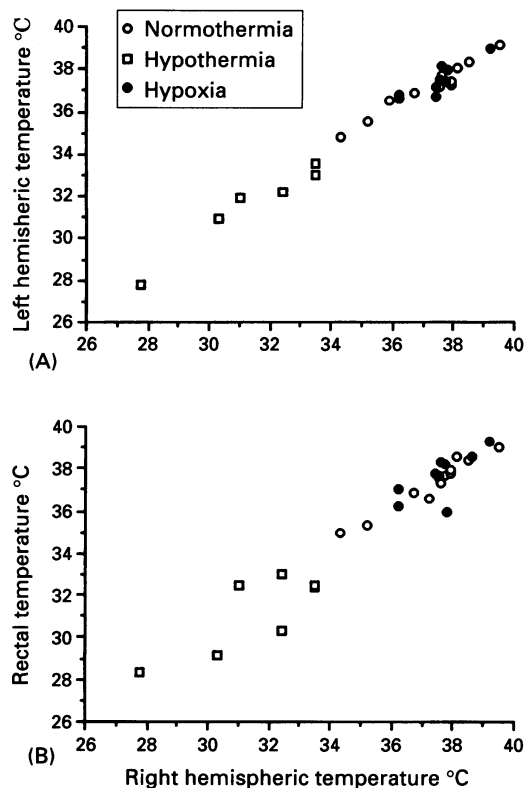


Figure 2 (A) shows the correlation between the right and left hemispheric temperatures ($r=0.99$) obtained from 12 animals in three different situations: normoxic normothermia (minimum 15 minutes), hypoxic normothermia (minimum 60 minutes), normoxic hypothermia (minimum 45 minutes). The mean values of the continuous recordings (sampled every minute) were compared. In (B) the correlation between the right hemispheric and the rectal temperature for the same situations is shown ($r=0.96$).

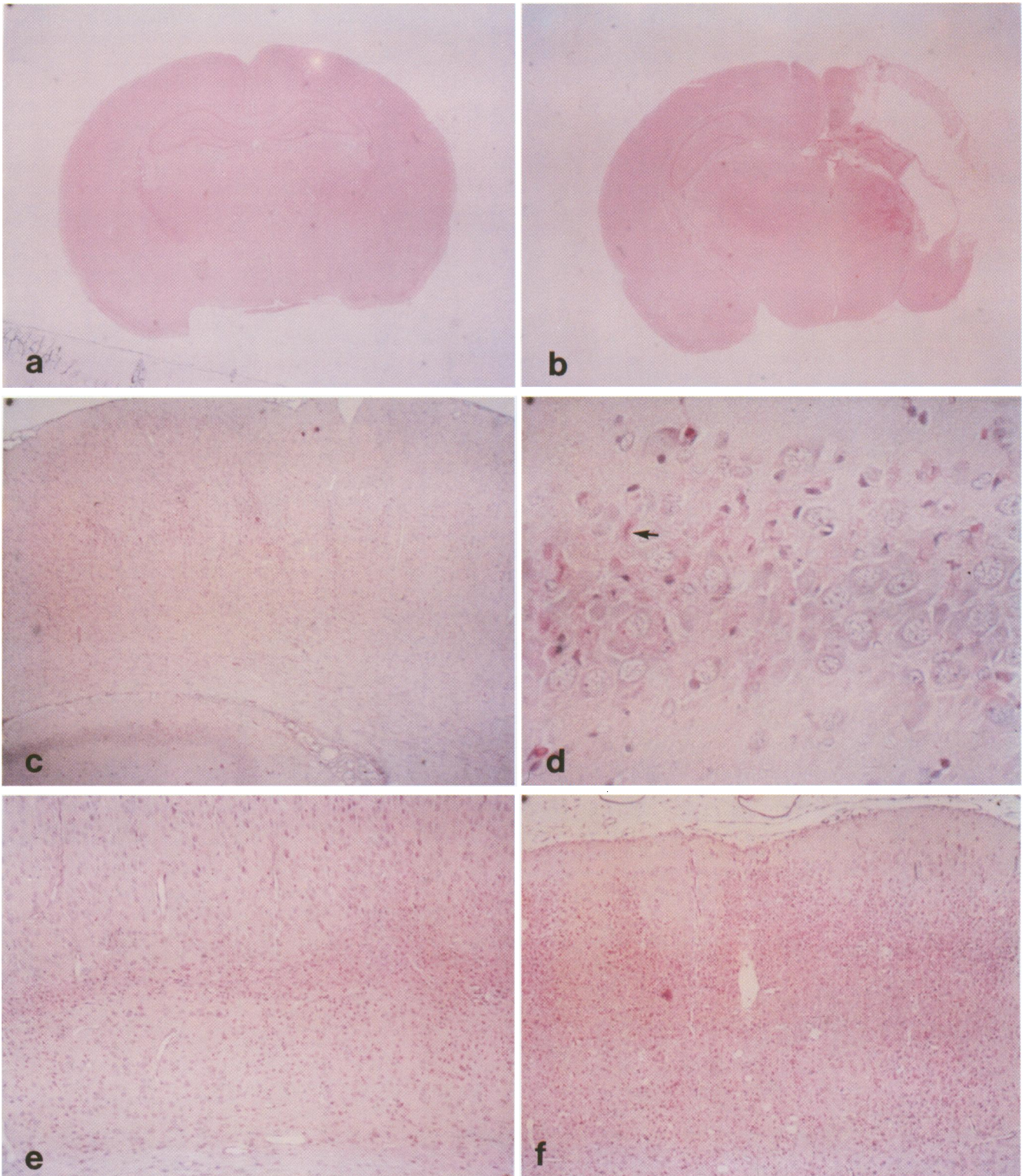


Figure 6A Coronal section through the mid-dorsal level of the hippocampus. The left common carotid artery had been permanently ligated. No damage was visible at this magnification (haematoxylin and eosin). At higher magnification all areas except the thalamus (which was undamaged) had a pathology score of 1 (figs 6C and D).

Figure 6B Coronal section through the mid-dorsal level of the hippocampus. The left common carotid artery was ligated. Complete infarction was evident, with disintegration of cerebral tissue supplied by the left middle cerebral artery (haematoxylin and eosin). The pathology score for all areas except the thalamus (which was 3) was 4.

Figure 6C Small localised areas with necrotic neurons in cortex, grade 1 damage (haematoxylin and eosin).

Figure 6D Hippocampal damage (CA2-CA3) grade 1, necrotic neurons with eosinophilic cytoplasm (arrow) (haematoxylin and eosin).

Figure 6E Cortex, grade 2 damage, with bandlike confluent infarcts in cortical layer 3 (haematoxylin and eosin).

Figure 6F Cortex, grade 3 damage, with even more confluent infarcts, but no disintegration of the tissue (haematoxylin and eosin).

inhibition of the production of oxygen free radicals,³⁷ of excitatory amino acids,³⁸ of lipid peroxidation³⁹ and postischaemic protein synthesis.⁴⁰ Similar studies have not been performed during postischaemic hypothermia.

Small differences in temperature during hypoxic exposure are critical to the extent of brain damage.⁴¹ We have shown that in this model rectal temperature is representative of brain temperature and we always used two animals with a permanent rectal probe as the 'thermometer' for the litter. During the current series of insults the rectal temperature was 38.3°C which is considered to be in the upper normal range for rat pups.⁴² Using this level of temperature during the insult mimics the clinical situation of perinatal asphyxia, where the fetal temperature is higher than that of the mother during parturition.⁴³

Hypothermia as treatment in this model implies separation from the dam for several hours and hypoglycaemia might cause additional damage. We therefore analysed blood glucose in treated and control animals and the concentrations were stable and normal during the whole study. Like others,⁴⁴ we found a moderate increase in glucose in response to hypothermia. Lactate values were normal and did not differ from control values at the end of hypothermia or normal temperature periods. The substantial base deficit (-14.3 mmol/l) at the end of hypoxia was fully compensated after three hours of hypothermia or normal temperature.

We were intrigued that Yager and Vannucci, who used the same model, found no protection with mild or moderate hypothermia.¹³ A significant difference in the experimental conditions between their and our study was that all their animals had a rectal probe in situ and were restrained. We hypothesise that the effect of stress has modulated the protective effect of hypothermia.³⁶

In this animal model of perinatal hypoxia-ischemia different ways of assessment of brain damage have been used: macroscopic reduction in hemispheric size,⁴⁵ morphological measurements of brain area,⁴⁶ weight estimation⁴⁷ as well as a neuropathological scoring system based on the degree and distribution of neuronal damage.¹⁶ We chose to do a detailed neuropathological assessment of the total hemisphere to evaluate the possibility of hypothermia changing the pattern and distribution of damage. This turned out not to be the case (fig 4). We used total cell counting in the hippocampus as a means of validating our pathology score. The subjective assessment and cell counting correlated well; $r=0.96$ (fig 5).

In conclusion, a reduction in rectal temperature of 6°C, starting after the insult and lasting for three hours, offers substantial protection against damage in all regions of the brain of the neonatal rat. Recently, in a more mature animal model of posthypoxia-ischaemia, the newborn pig,⁴⁸ we have also shown that 12 hours of mild posthypoxic hypothermia (4°C reduction) ameliorates secondary energy failure, as determined by ³¹P magnetic resonance

spectroscopy.⁴⁹ Before starting clinical trials of hypothermia following birth asphyxia, more animal work is needed to define the optimal duration and the effective 'time window' for this treatment, as well as its possible adverse effects. Hypothermia immediately after resuscitation might allow time for pharmacological agents to enter the brain and work at an earlier stage of the posthypoxic cascade of damaging processes.

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