Original Paper

Developmental Neuroscience

Dev Neurosci 2002;24:382–388
DOI: 10.1159/000069043

Received: June 8, 2002
Accepted: August 19, 2002

Neuroprotection of Creatine Supplementation in Neonatal Rats with Transient Cerebral Hypoxia-Ischemia

Kathryn H. Adcock[a] Johann Nedelcu[a, †] Thomas Loenneker[a] Ernst Martin[a]
Theo Wallimann[b] Bendicht P. Wagner[c]

[a]Neuroradiology and Magnetic Resonance Research, University Children's Hospital, [b]Department of Cell Biology, Swiss Federal Institute of Technology (ETH), Zurich, and [c]Pediatric Intensive Care, University Children's Hospital, Berne, Switzerland

Key Words
Hypoxia-ischemia · Neonatal rat · Neuroprotection · Creatine · Supplementation · Brain · Rat

Abstract
We hypothesized that creatine (Cr) supplementation would preserve energy metabolism and thus ameliorate the energy failure and the extent of brain edema seen after severe but transient cerebral hypoxia-ischemia (HI) in the neonatal rat model. Six-day-old (P6) rats received subcutaneous Cr monohydrate injections for 3 consecutive days (3 g/kg body weight/day), followed by ^{31}P-magnetic resonance spectroscopy (MRS) at P9. In a second group, P4 rats received the same Cr dose as above for 3 days prior to unilateral common carotid artery ligation followed 1 h later by 100 min of hypoxia (8% O_2) at P7. Rats were maintained at 37°C rectal temperature until magnetic resonance imaging was performed 24 h after HI. Cr supplementation for 3 days significantly increased the energy potential, i.e. the ratio of phosphocreatine to β-nucleotide triphosphate (PCr/βNTP) and PCr/inorganic phosphate (PCr/Pi) as measured by ^{31}P-MRS. Rats with hemispheric cerebral hypoxic-ischemic insult that had received Cr showed a significant reduction (25%) of the volume of edemic brain tissue compared with controls as calculated from diffusion-weighted images (DWI). Thus, prophylactic Cr supplementation demonstrated a significant neuroprotective effect 24 h after transient cerebral HI. We hypothesize that neuroprotection is probably due to the availability of a larger metabolic substrate pool leading to a reduction of the secondary energy failure because DWI has been reported to correlate with the PCr/Pi ratio in the acute phase of injury. Additional protection by Cr may be related to prevention of calcium overload, prevention of mitochondrial permeability transition pore opening and direct antioxidant effects.

Introduction

Perinatal cerebral hypoxia-ischemia (HI) leads to acute energy failure and cell death, resulting in chronic neurological symptoms. HI occurs in 2–4/1,000 full-term births and it is estimated that 10% of these infants will develop neurological disability such as cerebral palsy [1–3].

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0378–5866/02/0245–0382$18.50/0

Accessible online at:
www.karger.com/dne

Bendicht Wagner
Pediatric Intensive Care, Inselspital
CH–3010 Berne (Switzerland)
Tel. +41 31 632 9310, Fax +41 31 632 9748
E-Mail wagner@insel.ch

Studies of animal models of HI have shed some light onto the functional and temporal aspects of the pathogenesis of HI. A widely used model of neonatal HI is an adaptation of that developed by Levine [4], whereby unilateral common carotid artery ligation in the 7-day-old (P7) rat is followed by exposure to hypoxic conditions after a short recovery [5]. The model has a number of advantages: it represents a transient hemispheric cerebral insult reproducibly inducing a small core region within a large penumbra and is followed by the so-called secondary energy failure (SEF) [6, 7]. Histological and noninvasive magnetic resonance spectroscopy (MRS) and imaging (MRI) using this rat model have shown that there is, in fact, a multiphase pattern of the brain pathology [6–11]. (1) During HI, aerobic respiration fails; the end products of anaerobic respiration [creatine (Cr), inorganic phosphate (Pi) and lactate] exceed adenosine triphosphate (ATP) and phosphocreatine (PCr) and the pH falls [8, 11]. Hemispheric cytotoxic edema is visible on diffusion-weighted images (DWI) and apparent diffusion coefficient (ADC) maps, at a time when depletion of cellular energy stores causes neuronal swelling [6, 10]. (2) For a few hours after HI, energy metabolism recovers and cytotoxic edema vanishes except for a small core lesion in the parietal cortex [6, 11]. (3) However, a delayed and secondary rise in the levels of lactate, Cr and Pi and a fall in ATP and PCr begins within hours of the insult while brain pH and cerebral blood flow remain constant [8–11]. This phenomenon is called the SEF. It is associated with glial activation and a reappearance of edema, the progression of which can be traced with MRI [6]. Astrocytic swelling induces further cytotoxic edema, which is visible with DWI and ADC, whereas necrotic and apoptotic neurons enhance vasogenic edema, as is depicted by T_2-weighted imaging (T_2WI) [6, 7, 10]. (4) Abnormal histological findings, representing ongoing neurodegenerative cell death in the adult rat, continue for much longer than was previously thought [12, 13]. Eventually, the tissue is resorbed and any hope of recovery is lost.

In clinical settings, this multiphase pattern of brain pathology is also observed. After a transient cerebral HI insult, the fetus or newly born infant quickly recovers to a normal cardiovascular state. However, MRI and MRS examinations of the brain correspond to the SEF, and suggest, retrospectively, that perinatal cerebral HI had occurred [14–17]. In addition, the MRS observation of cerebral lactic alkalosis persisting months after HI may be indicative of an ongoing postasphyxial process, paralleling the slow progressing cell death in experimental models. Finally, the ratios and concentrations of several metabolites derived from spectroscopic investigations of asphyxiated infants within the first week of life have been found to correlate with their long-term outcome, such as the PCr/Pi ratio [14, 15, 18, 19], and the ATP/total exchangeable Pi and lactate/N-acetylaspartate ratios [16]. Therapeutic interventions aimed at lessening SEF may, therefore, reduce the long-term neuropsychological handicap caused by perinatal cerebral HI and may not share the fate of other neuroprotective agents, which were discontinued due to a lack of long-term improvements [3]. For example, postischemic hypothermia ameliorates the size of infarct and the drop in PCr/Pi in the neonatal rat and furthermore provides long-term improvement of the behavioral outcome [7, 20]. Other, more direct approaches to prevent energy metabolism failure may also prove beneficial after HI.

Cr is a simple guanidine compound, either synthesized endogenously from arginine, glycine and *S*-adenosylmethionine or ingested with fish and meat and is found throughout the body, including the brain [for a review, see ref. 21]. Its phosphorylated form, PCr, is the source of high-energy phosphate in the conversion of ADP to ATP by the enzyme Cr kinase at times of high cellular energy requirement [for a review, see ref. 22]. Cr supplementation has been shown to increase both Cr and PCr levels in the brain [23–26] and protect neurons [27], and provide functional benefits in several studies [28–32].

In light of these findings, we hypothesized that Cr supplementation would reduce the effect of SEF and the extent of brain edema observed after severe, transient cerebral HI in the neonatal rat model.

Methods

The animal studies reported here have been approved by the Animal Care and Experimentation Committees of the Cantons of Zurich and Berne, Switzerland. Guidelines for animal care, research and ethics published by the Swiss National Academy of Medical Sciences were strictly adhered to.

Cr Supplementation and ^{31}P-MRS

P6 Sprague Dawley rats of 16.5 ± 1.2 g (mean ± SD; n = 16) were injected subcutaneously with Cr monohydrate (3 g/kg body weight/day; 28 mg/ml in 0.9% sodium chloride) for 3 days. At P9, noninvasive ^{31}P-MRS under halothane anesthesia (4% induction, 1.5% maintenance in 70% N_2O, 30% O_2) was carried out with a purpose-built surface coil made to fit one hemisphere in a specially designed water-warmed rat holder. The ^{31}P spectra were recorded in pulse-acquire mode using an adiabatic 90° pulse, a bandwidth of 5 kHz, 512 signal averages and repetition time of 8 s. The spectra were fitted fully automatically in the time domain with prior knowledge using a combination of Lorentzian and Gaussian line shapes and 10-Hz line

broadening [6, 7]. Classical and clinically relevant indices of energy within the brain were calculated from the ratios of PCr to Pi or β-nucleotide triphosphate (βNTP) from the spectra [9, 14]. Saturation effects due to the different T_1 relaxation times of PCr (~4 s) or βNTP and Pi (~2 s) as reported elsewhere [33] do slightly underestimate the metabolite ratios, but do not affect the qualitative comparison of the ratios between control and Cr-supplemented animals.

Animal Model of HI

We used the modified Levine model of transient hemispheric cerebral HI [4, 5], which has been described in detail before [6, 10]. Briefly, P7 (n = 16) rats of 11.1 ± 0.7 g (mean ± SD), which had previously received Cr monohydrate supplementation as above for 3 days, were subjected to a right common carotid artery ligation under general anesthesia (halothane 4% induction, 1.5% maintenance in 70% N_2O, 30% O_2) and returned to their dams in a temperature-regulated cage for 1 h. The rats were then placed into a hypoxic chamber (8% O_2, 0.5% halothane) for 100 min during which time breathing was monitored. The chamber was also heated to ensure a constant body temperature of 37°C for the duration of the hypoxia. The rats were subsequently returned to their dams and maintained at 37°C in a specially designed cage for 24 h after the insult, at which time MRI was carried out.

It is our previous experience that P7 rats died 5 days after HI due to poor suckling [7, 20]. Although it was not our aim to maintain the rats for a long time, we routinely gavage fed HI rats every 6 h after HI in order to maintain their health for the duration of the experiment in accordance with the principle of permissive under-feeding in acute illness [34]. The feed consisted of 0.5 ml unsweetened condensed milk.

Magnetic Resonance Imaging

MRI was carried out in accordance with our previous studies [6, 20, 35]. Briefly, T_2WI was acquired with a multislice RARE technique [36] with TR = 4 s, RARE factor = 16 and an interecho interval of 22 ms, resulting in an effective echo time (TE_{eff}) of 252 ms. The matrix size was 256 × 128 (pixel dimensions 156 × 312 μm) and the field of view was 4 cm^2. A stimulated echo sequence was used to acquire DWI: TE = 18 ms, TR = 2 s, diffusion weighting factor b = 1,290 s/mm^2, matrix size = 128 × 64, field of view = 4 cm^2. The slice packages for both T_2WI and DWI comprised 8 1.5-mm-thick slices in the axial plane (coronal with respect to the rat) interleaved by an 0.3-mm gap, which encompassed the whole brain. All measurements were carried out on a 2-tesla (T) whole body MR system (Bruker Medical, Faellanden, Switzerland) equipped with an actively shielded gradient insert with a 33-cm bore, maximum gradient strength of 30 mT/m and 150 μs rise time. Fractional volume of tissue with edema was calculated from 8 DWI slices per animal as described previously [6, 7, 20]. Edema was defined as a hyperintense signal within the HI-affected hemisphere compared with the unaffected contralateral hemisphere. The area of edema from each slice was measured by automated software (Paravision, Bruker Medical) and summed to yield the entire edemic tissue volume, which was expressed as a fraction of whole brain volume.

Statistical Analysis

Comparisons between control and Cr-treated groups were carried out by two-sided exact tests (Mann-Whitney). All values are mean ± SEM.

Results

The rats tolerated subcutaneous injections well. There were no fatalities during the 3 days of injections, or during HI. However, there was 1 Cr-treated pup fatality due to aspiration after gavage feeding. Cr-supplemented rats did not differ from controls with respect to weight gain or rectal temperature (data not shown).

High-Energy Phosphorous-Containing Metabolite Ratios after Cr Supplementation

The levels of phosphorous-containing metabolites were measured in the hemisphere ipsilateral to the right common carotid artery ligation after 3 days of subcutaneous Cr supplementation in P9 rats. Figure 1 shows typical spectra for control and Cr-supplemented animals. The ratio of PCr/Pi was found to be 1.50 ± 0.12 (mean ± SEM) in control rats, but was significantly higher (2.25 ± 0.26) in Cr-supplemented animals (p = 0.028). The ratio of PCr/βNTP in control rats was 1.09 ± 0.14 (n = 8), which rose significantly to 1.52 ± 0.10 (n = 8) upon Cr supplementation (p = 0.021; fig. 2).

Brain Edema after Cr Supplementation

Figure 3 shows typical T_2WI and DWI obtained from P8 control or Cr-supplemented rats 24 h after HI. DWI and T_2WI hyperintensity represent cytotoxic and vasogenic edema, respectively. The MR images were hyperintense in the ipsilateral cortex, the hippocampus and basal ganglia in nonsupplemented animals, indicating widespread combined cytotoxic and vasogenic edema. Cytotoxic injury size was calculated to represent 42.5 ± 2.4% (n = 8) of total brain volume in control animals from DWI. In contrast, Cr-supplemented rats showed a marked reduction in the volume of tissue with edema, 32.3 ± 2.8% brain volume (n = 7) that was significantly different to controls (p = 0.0401; fig. 4). The secondary edema was mainly limited to the ipsilateral cortex in Cr-supplemented animals.

Discussion

We hypothesized that the subcutaneous Cr supplementation of neonatal rats would be neuroprotective after transient hemispheric HI. Indeed, it could be shown here that injection of Cr monohydrate for 3 days increased the PCr/βNTP and PCr/Pi ratios in the brain, by nearly 40 and 50%, respectively, in P9 animals compared with controls. Since the signal to noise ratio for βNTP and Pi

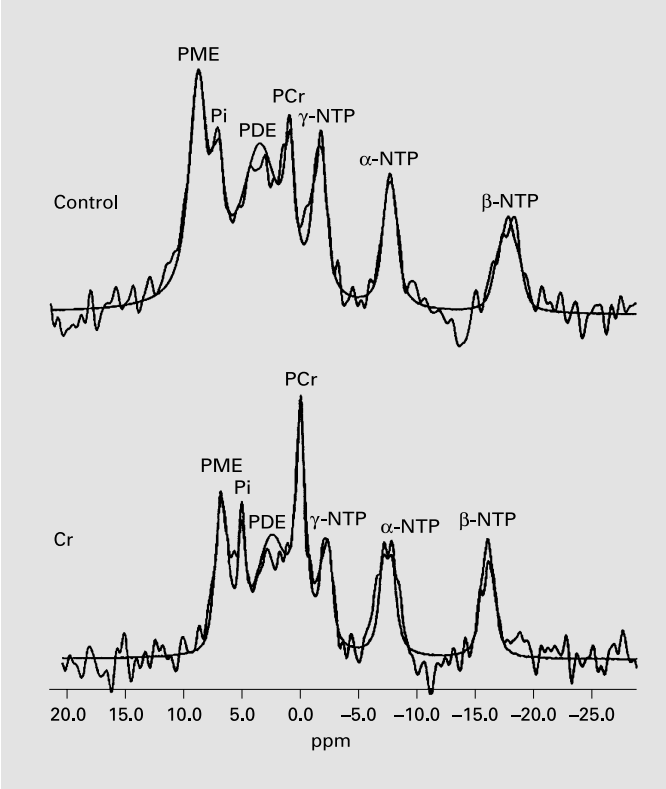

Fig. 1. Representative in vivo ^{31}P-MRS spectra of P9 rat brains from control (top) or Cr-supplemented (bottom) pups. PME = Phosphomonoester; PDE = phosphodiester. The smoothed lines in the figure represent spectra fitted fully automatically in the time domain with prior knowledge using a combination of Lorentzian and Gaussian line shapes and 10-Hz line broadening [6, 7].

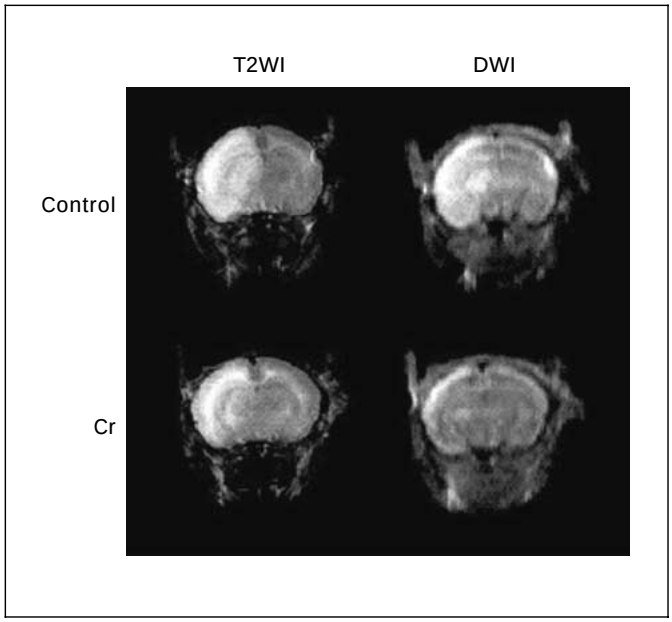

Fig. 3. T_2WIs (left-hand column) and DWIs (right-hand column) of 2 representative P8 rats 24 h after HI. The control animal (top) displayed marked DWI and T_2WI hyperintensity in the ipsilateral cortex, hippocampus and basal ganglia. In contrast, the Cr-supplemented animal (bottom) showed a marked reduction in the extent of hyperintensity in MR images; while the ipsilateral cortex was affected by HI, the hippocampus and basal ganglia were spared from edema.

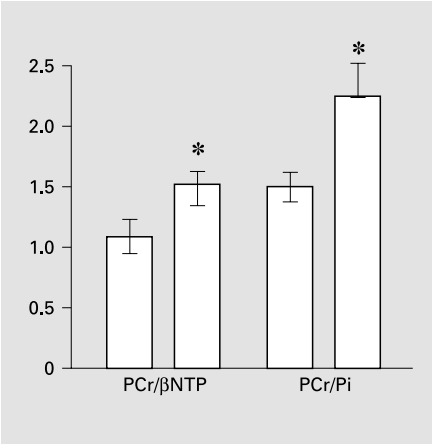

Fig. 2. Changes in PCr/βNTP and PCr/Pi after 3 days of Cr supplementation in P9 rats. Open bars = Control; filled bars = Cr-supplemented rats. Cr significantly increased both PCr/βNTP and PCr/Pi compared with control animals. (* $p < 0.05$).

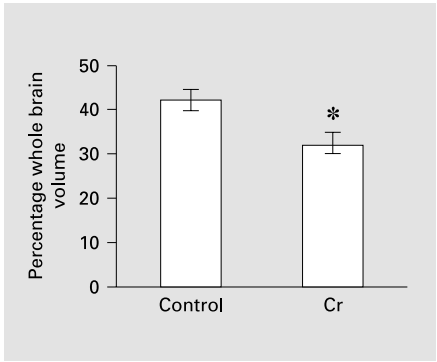

Fig. 4. Changes in volume of tissue with edema in P8 rats 24 h after HI subsequent to 3 days Cr supplementation. Open bar = Control + HI rats; filled bar = Cr-supplemented + HI rats. Cr significantly decreased the size of cytotoxic edema compared with control rats (* $p < 0.05$).

remained almost constant between supplemented and nonsupplemented animals, the increase in the PCr/βNTP and PCr/Pi ratios appeared to be due to an increase in PCr within the brain. Furthermore, it is shown here that Cr-supplemented rats had 25% smaller HI lesions 24 h after HI compared with nonsupplemented litter mates. These data suggest that after just 3 days of Cr supplementation, sufficient Cr was able to cross the blood brain barrier to ameliorate the severe damage inflicted by HI.

Effects of Cr Supplementation on Brain Metabolism

^{1}H-MRS reveals that 4 weeks of oral supplementation increases total Cr in the adult human brain [37]. Cr has also been used successfully in the treatment of infants with Cr deficiency; after oral supplementation, not only was there a significant increase in cerebral Cr concentration, but also improvements of neurological symptoms [38]. To date, there is no literature on the effects of exogenous Cr on newborn infants.

We have shown here that PCr/Pi and PCr/βNTP increase upon Cr administration from P6 to P8. In addition, we have found increased PCr/Pi and PCr/βNTP after Cr injections in a small group of younger rats (P4–P6, data not shown). These data are in agreement with the observation that Cr supplementation increases PCr/βNTP in P10 rats [25]. It is interesting to note that augmentation of the energetic state by Cr is lost by P20 [25], which may be related to the fact that the Cr transporter is significantly downregulated during brain differentiation [39]. Other studies of Cr supplementation for more extended periods of time have shown increases, albeit small ones, with variability in different brain regions, of both total Cr and PCr in the brain of adult rabbits, rats, mice and guinea pigs [26, 30, 40]. This suggests that younger, immature rats are more responsive to Cr supplementation, perhaps due to a higher expression of the Cr transporter [39]. Since P7 rats are, in many ways, equivalent to near-term humans with respect to metabolism and maturation of the brain [41], we speculate that Cr supplementation may be very effective in increasing cerebral PCr in human newborns as well. The rate of reaction of Cr kinase, i.e. the interconversion of PCr and ATP in the brain is not significantly altered by Cr injections in the immature rat and rabbit, suggesting that excess Cr is sufficient to exert the functional consequences of Cr supplementation [25, 26].

Effects of Prophylactic Cr Supplementation on Cerebral HI

DWI hyperintensity returns 2 h after HI in the neonatal rat and reaches a maximum between 20 and 30 h [6, 35]. The secondary edema profile shown here at 24 h after HI correlated well with previous studies [6, 7, 20, 35]. Therefore, 24 h after the HI insult, we would expect to see the effect of Cr supplementation on cytotoxic edema. Indeed, Cr significantly reduced the extent of hyperintensity seen in DWI at this time. We have previously shown that DWI hyperintensity is proportional to the extent of the reduction in PCr/Pi, i.e. the SEF [6]. Given the correlation between DWI and PCr/Pi during SEF, we suspect that Cr acts by lessening the SEF due to the increased preischemic PCr/Pi observed in age-matched rats. Thus, reduction of SEF is likely to be due to the availability of a large substrate pool as the neuroprotective source. In addition, there is a linear relationship between the cerebral ADC of water and PCr/Pi and hence SEF [42]. DWI is, therefore, an appropriate tool for measuring SEF after HI; moreover, DWI has a shorter sample time, a higher spatial resolution and shows fewer movement artifacts than ^{31}P-MRS.

In concert with increased PCr/βNTP and PCr/Pi, Cr administration may affect energy metabolism by enhancing mitochondria function via Cr-stimulated respiration [43]. Cr administration also increases the ADP sensitivity of mitochondrial respiration [44]; ADP clearly increases as ATP is hydrolyzed in times of energy crises, e.g. during SEF. The *mdx* mouse has impaired energy metabolism in muscle cells and is routinely used as a model of Duchenne muscular dystrophy. If these mice are fed a Cr-enriched diet, their mitochondrial function is rescued and elevated to wild-type levels [45]. In addition, the energetics of calcium handling in *mdx* myotubes was improved [46] and calcium-induced muscle necrosis significantly reduced [45]. Upon energy failure, cells usually display increasing difficulties with calcium handling, which may eventually lead to chronic calcium overload, a condition shown to result in further deterioration of cellular energy status, in calcium uptake by mitochondria and opening of the mitochondrial permeability transition pore [47]. It has been shown, with transgenic mice expressing mitochondrial Cr kinase in their liver mitochondria, that Cr protected these mitochondria from undergoing calcium-induced permeability transition pore opening [48], an early event in apoptosis. Therefore, it has been proposed that Cr could protect mitochondria from failure by stabilizing the multienzyme complex of mitochondrial Cr kinase, porin and ATP/ADP translocase [49], thus providing continued

transport of high-energy phosphate into the cytoplasm. It is possible that in our model of SEF, Cr could be acting in such a manner. Finally, recently discovered properties of Cr are that it can directly act as an antioxidant against aqueous reactive oxygen species, e.g. peroxynitrite in vitro [50] or as a protective agent against excitotoxic damage of neurons [27, 51]. Intracellular calcium overload, free radicals and excitatory amino acids have been found to play an important role in brain injury following HI.

It is possible that Cr supplementation would have long-lasting effects. There is a strong correlation between SEF and the pathological outcome; the minimum value of PCr/Pi is proportional to the severity of the infarct [52]. Despite there being no long-term animal investigations into the relationship between SEF and the neurological outcome, reports from the clinic indicate that the severity of SEF (i.e. PCr/Pi) in the first weeks after birth correlate with the neurological outcome [14–16, 53, 54]. Therefore, we predict that by reducing the extent of SEF, one would see an improvement of neurological symptoms both in the short and long term.

Holtzman et al. [25] have shown that subcutaneous injection of Cr into the 10-day-old rat, a time when energy metabolism is immature, increases PCr/βNTP, survival and prevents hypoxia-induced seizures. Feeding Cr-enriched chow to pregnant mice increased ATP concentrations in brain stem slices of the neonates, which was sufficient to attenuate both firing duration and strength of hypoglossal neurons during and after anoxia in vitro [55, 56]. Adult rats, despite having a slight increase in overall Cr after supplementation, do not show a significant improvement of ADC during transient global hypoxia [24, 57], suggesting again that the metabolic maturity of the animal is critical to its response to Cr and HI.

In concert with our findings of neuroprotection, Cr supplementation has shown potential benefits in animal models of brain pathologies other than HI, such as amyotrophic lateral sclerosis [28], Parkinson's disease [29], Huntington's disease [30, 31, 51] and traumatic brain injury [32].

We have shown that metabolically immature rats benefit from Cr supplementation by exhibiting an increased PCr/βNTP ratio, indicating that an elevation of high-energy phosphates in the brain seems to be able to protect the brain after HI in the newborn rat. The long-term benefits of Cr supplementation are currently under investigation prior to evaluation of its effect in the clinical setting. It is our belief that supplementation with exogenous Cr during and after HI, by preserving brain energy levels, with its positive consequences on calcium homeostasis and mitochondrial function, could limit, if not prevent, damage caused by HI by ameliorating SEF.

Acknowledgements

This work was supported by the Swiss National Science Foundation (project No. 32-52647-97 and 32-64110-00).

References

1 Patel J, Edwards AD: Prediction of outcome after perinatal asphyxia. Curr Opin Pediatr 1997;9:128–132.
2 Thornberg E, Thiringer K, Odeback A, Milsom I: Birth asphyxia: Incidence, clinical course and outcome in a Swedish population. Acta Paediatr 1995;84:927–932.
3 Vannucci RC, Perlman JM: Interventions for perinatal hypoxic-ischemic encephalopathy. Pediatrics 1997;100:1004–1014.
4 Levine S: Anoxic-Ischemic encephalopathy in rats. Am J Pathol 1960;36:1–17.
5 Rice JE 3rd, Vannucci RC, Brierley JB: The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol 1981;9:131–141.
6 Nedelcu J, Klein MA, Aguzzi A, et al: Biphasic edema after hypoxic-ischemic brain injury in neonatal rats reflects early neuronal and late glial damage. Pediatr Res 1999;46:297–304.
7 Nedelcu J, Klein MA, Aguzzi A, Martin E: Resuscitative hypothermia protects the neonatal rat brain from hypoxic-ischemic injury. Brain Pathol 2000;10:61–71.
8 Palmer C, Brucklacher RM, Christensen MA, Vannucci RC: Carbohydrate and energy metabolism during the evolution of hypoxic-ischemic brain damage in the immature rat. J Cereb Blood Flow Metab 1990;10:227–235.
9 Lorek A, Takei Y, Cady EB, et al: Delayed ('secondary') cerebral energy failure after acute hypoxia-ischemia in the newborn piglet: Continuous 48-hour studies by phosphorus magnetic resonance spectroscopy. Pediatr Res 1994;36:699–706.
10 Rumpel H, Buchli R, Gehrmann J, et al: Magnetic resonance imaging of brain edema in the neonatal rat: A comparison of short- and long-term hypoxia-ischemia. Pediatr Res 1995;38:113–118.
11 Penrice J, Lorek A, Cady EB, et al: Proton magnetic resonance spectroscopy of the brain during acute hypoxia-ischemia and delayed cerebral energy failure in the newborn piglet. Pediatr Res 1997;41:795–802.
12 Coimbra C, Drake M, Boris-Moller F, Wieloch T: Long-lasting neuroprotective effect of post-ischemic hypothermia and treatment with an anti-inflammatory/antipyretic drug. Evidence for chronic encephalopathic processes following ischemia. Stroke 1996;27:1578–1585.
13 Corbett D, Hamilton M, Colbourne F: Persistent neuroprotection with prolonged postischemic hypothermia in adult rats subjected to transient middle cerebral artery occlusion. Exp Neurol 2000;163:200–206.
14 Cady EB: Magnetic resonance spectroscopy in neonatal hypoxic-ischaemic insults. Childs Nerv Syst 2001;17:145–149.
15 Martin E, Buchli R, Ritter S, et al: Diagnostic and prognostic value of cerebral ^{31}P magnetic resonance spectroscopy in neonates with perinatal asphyxia. Pediatr Res 1996;40:749–758.

16 Penrice J, Cady EB, Lorek A, et al: Proton magnetic resonance spectroscopy of the brain in normal preterm and term infants, and early changes after perinatal hypoxia-ischemia. Pediatr Res 1996;40:6–14.
17 Robertson RL, Maier SE, Robson CD, et al: MR line scan diffusion imaging of the brain in children. AJNR Am J Neuroradiol 1999;20: 419–425.
18 Azzopardi D, Wyatt JS, Hamilton PA, et al: Phosphorus metabolites and intracellular pH in the brains of normal and small for gestational age infants investigated by magnetic resonance spectroscopy. Pediatr Res 1989;25:440–444.
19 Hope PL, Costello AM, Cady EB, et al: Cerebral energy metabolism studied with phosphorus NMR spectroscopy in normal and birth-asphyxiated infants. Lancet 1984;ii:366–370.
20 Wagner BP, Nedelcu J, Martin E: Delayed postischemic hypothermia improves long-term behavioral outcome after cerebral hypoxia-ischemia in neonatal rats. Pediatr Res 2002;51: 354–360.
21 Persky AM, Brazeau GA: Clinical pharmacology of the dietary supplement creatine monohydrate. Pharmacol Rev 2001;53:161–176.
22 Wyss M, Kaddurah-Daouk R: Creatine and creatine metabolism. Physiol Rev 2000;80: 1107–1213.
23 Balestrino M, Rebaudo R, Lunardi G: Exogenous creatine delays anoxic depolarization and protects from hypoxic damage: Dose-effect relationship. Brain Res 1999;816:124–130.
24 Michaelis T, Wick M, Fujimori H, et al: Proton MRS of oral creatine supplementation in rats. Cerebral metabolite concentrations and ischemic challenge. NMR Biomed 1999;12:309–314.
25 Holtzman D, Togliatti A, Khait I, Jensen F: Creatine increases survival and suppresses seizures in the hypoxic immature rat. Pediatr Res 1998;44:410–414.
26 Holtzman D, Khait I, Mulkern R, et al: In vivo development of brain phosphocreatine in normal and creatine-treated rabbit pups. J Neurochem 1999;73:2477–2484.
27 Brewer GJ, Wallimann TW: Protective effect of the energy precursor creatine against toxicity of glutamate and beta-amyloid in rat hippocampal neurons. J Neurochem 2000;74:1968–1978.
28 Klivenyi P, Ferrante RJ, Matthews RT, et al: Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. Nat Med 1999;5:347–350.
29 Matthews RT, Ferrante RJ, Klivenyi P, et al: Creatine and cyclocreatine attenuate MPTP neurotoxicity. Exp Neurol 1999;157:142–149.
30 Matthews RT, Yang L, Jenkins BG, et al: Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. J Neurosci 1998;18:156–163.
31 Shear DA, Haik KL, Dunbar GL: Creatine reduces 3-nitropropionic-acid-induced cognitive and motor abnormalities in rats. Neuroreport 2000;11:1833–1837.
32 Sullivan PG, Geiger JD, Mattson MP, Scheff SW: Dietary supplement creatine protects against traumatic brain injury. Ann Neurol 2000;48:723–729.
33 Gruetter R, Fusch C, Martin E, Boesch C: Determination of saturation factors in ^{31}P NMR spectra of the developing human brain. Magn Reson Med 1993;29:7–11.
34 Zaloga GP, Roberts P: Permissive underfeeding. New Horiz 1994;2:257–263.
35 Rumpel H, Nedelcu J, Aguzzi A, Martin E: Late glial swelling after acute cerebral hypoxia-ischemia in the neonatal rat: A combined magnetic resonance and histochemical study. Pediatr Res 1997;42:54–59.
36 Hennig J, Nauerth A, Friedburg H: RARE imaging: A fast imaging method for clinical MR. Magn Reson Med 1986;3:823–833.
37 Dechent P, Pouwels PJ, Wilken B, et al: Increase of total creatine in human brain after oral supplementation of creatine-monohydrate. Am J Physiol 1999;277:R698–R704.
38 Stockler S, Holzbach U, Hanefeld F, et al: Creatine deficiency in the brain: A new, treatable inborn error of metabolism. Pediatr Res 1994;36:409–413.
39 Kekelidze T, Khait I, Togliatti A, Holtzman D: Brain creatine kinase and creatine transporter proteins in normal and creatine-treated rabbit pups. Dev Neurosci 2000;22:437–443.
40 Ipsiroglu OS, Stromberger C, Ilas J, et al: Changes of tissue creatine concentrations upon oral supplementation of creatine-monohydrate in various animal species. Life Sci 2001;69: 1805–1815.
41 Hagberg H, Bona E, Gilland E, Puka-Sundvall M: Hypoxia-ischaemia model in the 7-day-old rat: Possibilities and shortcomings. Acta Paediatr Suppl 1997;422:85–88.
42 Thornton JS, Ordidge RJ, Penrice J, et al: Temporal and anatomical variations of brain water apparent diffusion coefficient in perinatal cerebral hypoxic-ischemic injury: Relationships to cerebral energy metabolism. Magn Reson Med 1998;39:920–927.
43 Kay L, Nicolay K, Wieringa B, et al: Direct evidence for the control of mitochondrial respiration by mitochondrial creatine kinase in oxidative muscle cells in situ. J Biol Chem 2000;275: 6937–6944.
44 Walsh B, Tonkonogi M, Soderlund K, et al: The role of phosphorylcreatine and creatine in the regulation of mitochondrial respiration in human skeletal muscle. J Physiol 2001;537: 971–978.
45 Passaquin AC, Renard M, Kay L, et al: Creatine supplementation reduces skeletal muscle degeneration and enhances mitochondrial function in *mdx* mice. Neuromuscul Disord 2002;12:174–182.
46 Pulido SM, Passaquin AC, Leijendekker WJ, et al: Creatine supplementation improves intracellular Ca^{2+} handling and survival in *mdx* skeletal muscle cells. FEBS Lett 1998;439:357–362.
47 Kruman II, Mattson MP: Pivotal role of mitochondrial calcium uptake in neural cell apoptosis and necrosis. J Neurochem 1999;72:529–540.
48 O'Gorman E, Beutner G, Dolder M, et al: The role of creatine kinase in inhibition of mitochondrial permeability transition. FEBS Lett 1997;414:253–257.
49 Schlattner U, Forstner M, Eder M, et al: Functional aspects of the X-ray structure of mitochondrial creatine kinase: A molecular physiology approach. Mol Cell Biochem 1998;184: 125–140.
50 Lawler JM, Barnes WS, Wu G, et al: Direct antioxidant properties of creatine. Biochem Biophys Res Commun 2002;290:47–52.
51 Malcon C, Kaddurah-Daouk R, Beal MF: Neuroprotective effects of creatine administration against NMDA and malonate toxicity. Brain Res 2000;860:195–198.
52 Blumberg RM, Cady EB, Wigglesworth JS, et al: Relation between delayed impairment of cerebral energy metabolism and infarction following transient focal hypoxia-ischaemia in the developing brain. Exp Brain Res 1997;113: 130–137.
53 Azzopardi D, Wyatt JS, Cady EB, et al: Prognosis of newborn infants with hypoxic-ischemic brain injury assessed by phosphorus magnetic resonance spectroscopy. Pediatr Res 1989;25: 445–451.
54 Hanrahan JD, Cox IJ, Azzopardi D, et al: Relation between proton magnetic resonance spectroscopy within 18 hours of birth asphyxia and neurodevelopment at 1 year of age. Dev Med Child Neurol 1999;41:76–82.
55 Wilken B, Ramirez JM, Probst I, et al: Creatine protects the central respiratory network of mammals under anoxic conditions. Pediatr Res 1998;43:8–14.
56 Wilken B, Ramirez JM, Probst I, et al: Anoxic ATP depletion in neonatal mice brainstem is prevented by creatine supplementation. Arch Dis Child Fetal Neonatal Ed 2000;82:F224–F227.
57 Wick M, Fujimori H, Michaelis T, Frahm J: Brain water diffusion in normal and creatine-supplemented rats during transient global ischemia. Magn Reson Med 1999;42:798–802.