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Effects of Allopurinol and Deferoxamine on Reperfusion Injury of the Brain in Newborn Piglets after Neonatal Hypoxia-Ischemia

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ABSTRACT

The hypothesis was tested that treatment with allopurinol, a xanthine oxidase inhibitor, or deferoxamine, a chelator of non-protein-bound iron, preserved cerebral energy metabolism, attenuated development of edema, and improved histologic outcome in the newborn piglet at 24 h after hypoxia-ischemia. Thirty-two newborn piglets were subjected to 1 h of hypoxia-ischemia by occluding both carotid arteries and reducing the fraction of inspired oxygen; five newborn piglets served as sham-operated controls. The depth of hypoxia-ischemia was controlled by phosphorous magnetic resonance spectroscopy. Upon reperfusion and reoxygenation, piglets received vehicle ($n = 12$), allopurinol (30 mg/kg/d, $n = 10$), or deferoxamine (12.5 mg/kg/d, $n = 10$). The cerebral energy status was determined with phosphorous magnetic resonance spectroscopy. The presence of vasogenic edema was assessed by T2-weighted magnetic resonance imaging. Brain cell injury was assessed with caspase-3 activity, histology, and terminal deoxynucleotidyl transferase-mediated dUTP-biotin *in situ* nick end (TUNEL)-labeling. At 24 h after hypoxia-ischemia, the phosphocreatine/inorganic phosphate ratios were significantly decreased in vehicle-treated, but not in allopurinol- or deferoxamine-treated piglets. Water T2 values were significantly increased at 24 h after hypoxia-ischemia in cerebral cortex, thalamus, and striatum of vehicle-treated piglets, but not in allopurinol- and deferoxamine-treated piglets. No differences in caspase-3 activity, histologic outcome, or TUNEL-labeling were demonstrated between the three treatment groups. We suggest that allopurinol and deferoxamine may have an additional value in the treatment of perinatal hypoxia-ischemia with other neuroprotective agents or in combination with hypothermia. **(*Pediatr Res* 54: 516–522, 2003)**

Abbreviations

BE, base excess
Fio_2, fraction of inspired oxygen
NPBI, nonprotein-bound iron
MABP, mean arterial blood pressure
MRI, magnetic resonance imaging
PCr, phosphocreatine
Pi, inorganic phosphate
^{31}P-MRS, phosphorous magnetic resonance spectroscopy
TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin *in situ* nick end labeling

The neonatal brain appears to be vulnerable to oxidative stress after perinatal hypoxia-ischemia with reperfusion and reoxygenation due to excessive free radical production, relatively large amounts of NPBI production, and inadequate scavenging mechanisms to counteract these potentially neurotoxic events (1). During perinatal hypoxia-ischemia and upon reperfusion, a biochemical cascade occurs, including modification of the NMDA receptor-ion channel complex, leading to increased intracellular Ca^{2+} and resulting in the conversion of xanthine dehydrogenase to xanthine oxidase (2). During the primary hypoxic-ischemic insult, ADP is degraded to hypoxanthine and then oxidated to xanthine and on to uric acid by xanthine oxidase upon reperfusion [for review, see Fellman and Raivio (3)]. During these reactions, superoxide and hydrogen peroxide are formed, which can be converted to the highly reactive hydroxyl radical through the Haber-Weiss reaction, catalyzed by ferrous iron (4). The reactive oxygen species

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produced in this way can result in modification of the mitochondrial membrane, eventually leading to cell membrane and DNA damage, cytochrome *c* leakage, caspase activation, and DNA fragmentation, and, subsequently, to secondary cell death.

After hypoxia-ischemia, secondary energy failure occurs from about 6 h after the insult, depending on the duration and intensity of the hypoxic-ischemic insult (5). This process can be studied with ^{31}P-MRS, as performed earlier in a newborn piglet model of perinatal asphyxia (6). Previous studies in animals and neonates showed that treatment with allopurinol, a xanthine oxidase inhibitor, upon reperfusion preserved the cerebral hemodynamics and electrocortical brain activity and showed neuroprotective effects (7–10). Short-term survival studies in newborn lambs and in dogs demonstrated beneficial effects of deferoxamine, a chelator of NPBI, on the amplitude-integrated EEG, cerebral blood flow, and metabolic decay (7, 11). However, effects of allopurinol and deferoxamine on cerebral energy status, amount of vasogenic edema, and histology at 24 h after the hypoxic-ischemic insult have not been studied previously. We hypothesized that allopurinol and deferoxamine had neuroprotective effects in a piglet model of perinatal hypoxia-ischemia with respect to the beforementioned parameters.

MATERIALS AND METHODS

Animal preparation and instrumentation. Thirty-two newborn Dutch store piglets, gestational age 115 ± 1 d (mean ± SEM), weight 1.69 ± 0.16 kg, were used with a postnatal age range from 1 to 3 d (12). Anesthesia was induced with 4% isoflurane in a N_2O/O_2 mixture (79%/21%). After intubation, the piglets were mechanically ventilated using a continuous flow, pressure-controlled ventilator (Amsterdam infant ventilator, HoekLoos, Amsterdam, the Netherlands). Anesthesia was maintained during the procedure using 1.5% isoflurane in the same N_2O/O_2 mixture. Venous catheters were inserted for continuous infusion of glucose 5%/NaCl 0.45% (5 mL/kg/h) and drug infusion. Before each skin incision, local anesthesia with lidocaine 1% was applied. A catheter was advanced in the right femoral artery for continuous measurement of the MABP, and the blood was heparinized [5 units (U)/h]. Remotely inflatable vascular cuffs (OC2a, *In vivo* Metric, Healdsburg, CA, U.S.A.) were placed around both common carotid arteries at the level of the thyroid cartilage. Amoxicillin (100 mg/kg/d), gentamicin (5 mg/kg/d), and atropine (0.01 mg/kg) were administered intravenously. During the experiment, rectal temperature was measured and maintained between 38° and 39°C using a heat lamp and/or water blanket.

The Animal Research Committee of the Utrecht University, the Netherlands, approved experimental protocols.

Experimental protocol. After completion of the surgical procedure, the piglets were allowed to achieve hemodynamic stability. Thereafter, the piglets were transferred to the MR unit for baseline ^{31}P-MRS measurements. During these measurements, anesthesia was maintained with 1.5% isoflurane in an O_2/N_2O mixture (30%/70%). Animals were paralyzed with pancuronium bromide (0.25 mg/kg i.v.). Piglets were subjected to 1 h of hypoxia-ischemia by inflating the occluders surrounding both common carotid arteries and reducing the Fio_2 until the PCr/Pi ratios had decreased to at least 30% of baseline values, as guided by frequent ^{31}P-MRS measurements, for approximately 45 min of the 1-h hypoxic-ischemic period. When severe systemic hypotension (MABP <30 mm Hg) or bradycardia developed, the Fio_2 was minimally increased to prevent further decrease of blood pressure or to obtain recovery of the heart rate. Isoflurane administration was discontinued from 10 min after start of hypoxia-ischemia until 10 min after reperfusion to reduce the effect of anesthetics on cardiovascular stability during the actual insult, but the administration of N_2O was continued. After 1 h of hypoxia-ischemia, the occluders were deflated and the oxygen intake increased to obtain normoxia (partial pressure of arterial oxygen, 80–120 mm Hg), carefully avoiding hyperoxia. The piglets were randomly assigned to receive vehicle (5 mL/kg i.v. 0.9% NaCl); allopurinol (Multipharma, Weesp, the Netherlands), 20 mg/kg upon reperfusion and a repeated dose of 10 mg/kg at 12 h; or deferoxamine (Novartis, Basel, Switzerland), 10 mg/kg upon reperfusion and a repeated dose of 2.5 mg/kg at 12 h, injected intravenously by a perfusor pump over 15 min. Five additional piglets served as sham-operated controls and were not subjected to hypoxia-ischemia. MABP, heart rate, oxygen saturation using a pulse oxymeter (Nellcor NPB-290, Tyco Healthcare, Pleasanton, CA, U.S.A.), and rectal temperature were measured during the study protocol. Sampling of arterial blood gases, arterial pH, and glucose was performed at baseline, at the end of 1 h of hypoxia-ischemia, and at 24 h of reperfusion. Between 3 h and 24 h after the start of hypoxia-ischemia, the piglets were observed in the piglet intensive care unit. At 24 h after reperfusion, the piglets were transferred to the MR unit and ^{31}P-MRS measurements were repeated.

^{31}P-MRS. ^{31}P-MRS experiments were performed at 81 MHz on an INOVA console (Varian, Palo Alto, CA, U.S.A.) interfaced to a 4.7 T Oxford magnet with a bore size of 40 cm. A ∅ 4-cm surface coil was used for signal excitation and nonlocalized detection and placed onto the intact scalp over the parietal lobes. A 1.0-ms adiabatic half passage pulse was used for excitation. To minimize T1 effects, a repetition time of 10 s was used. Thirty-two acquisitions were averaged at baseline and during reperfusion and eight acquisitions during hypoxia-ischemia to evaluate the depth of hypoxia-ischemia. Peak amplitudes of PCr and Pi were determined with time-domain fitting procedures using prior knowledge (Variable Projection Method; Magnetic Resonance User Interface 97.2, Universitat Autònoma de Barcelona, Barcelona, Spain) (13).

Metabolite ratios of PCr/Pi were calculated. To determine whether the decline in the energy status during the 1-h period of hypoxia-ischemia was the same for the different groups, areas under the PCr/Pi curve were determined for each piglet (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA, U.S.A.).

T2-weighted MRI. T2-weighted multislice spin-echo MRI was performed at baseline and at 24 h after hypoxia-ischemia using a birdcage volume coil. Repetition time was 3 s; echo times were 25, 40, and 70 ms; and a slice sickness of 2 mm, 16 slices and two transitions, was used. Quantitative T2 maps were generated from these T2-weighted MR images and time course changes of T2 relaxation times were assessed in brain areas known to be sensitive to hypoxia-ischemia, *e.g.* cortex, striatum, thalamus and white matter, at baseline and 24 h after hypoxia-ischemia. These regions

of interest were manually drawn in the T2 maps to average regional T2 values, with the use of the image analysis software package Image Browser (Varian).

Allopurinol and oxypurinol levels in serum. Serum samples were taken at 0.5, 1.5, 2, 3, and 12 h after the first gift of allopurinol and 0.5 and 12 h after the second gift of allopurinol for assessment of allopurinol and oxypurinol levels. For the analysis of serum and brain tissue, a modified reversed-phase high-pressure liquid-chromatographic method with UV-detection according to Wung and Howel (14) was used.

Assay of caspase-3 like activity. The experiment was ended at 24 h after hypoxia-ischemia by an overdose of pentobarbital, and the brain was rapidly perfused with normal saline to remove the excess of blood. Right hemispheres of all piglets were dissected into cortex, striatum, thalamus, and white matter and were snap frozen in liquid nitrogen and stored at −80°C until further analysis. Tissue pieces were homogenized by sonication in 10 volumes of ice-cold 50 mM Tris-HCl (pH 7.3), containing 5 mM EDTA, aliquoted and stored at −80°C. The protein concentrations were determined according to Whitaker and Granum (15), adapted for microplates using a Spectramax Plus plate reader (Molecular Devices, Menlo Park, CA, U.S.A.). Samples of homogenate (25 μL) were mixed with 75 μL of extraction buffer, containing 50 mM Tris-HCl (pH 7.3), 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM NaN3, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, 2.5 μg/mL leupeptin, 10 μg/mL aprotinin, and 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), on a microtiter plate (Microfluor; Dynatech Labs, Chantilly, VA, U.S.A.). After incubation for 15 min at room temperature, 100 μL of peptide substrate, 50 μM Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC, from Enzyme Systems Products, Livermore, CA, U.S.A.) in extraction buffer without inhibitors or CHAPS, but with 4 mM DTT, was added. Cleavage of DEVD-AMC was measured at 37°C using a Spectramax Gemini microplate fluorometer (Molecular Devices) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm, and expressed as picomoles AMC released per milligram protein and per minute.

NPBI levels in serum and brain. NPBI plasma levels at baseline and at 24 h after hypoxia-ischemia were detected by HPLC using the method described by Kime *et al.* (16) partially modified. The system was operated isocritically at a pressure of approximately 115 Bar and flow of 0.75 mL/min. The detection wavelength was 450 nm with a reference wavelength at 620 nm. A low-affinity ligand, disodium nitryloacetic acid, was first used to complex all low-molecular-weight iron and iron non-specifically bound to serum proteins such as albumin. Because it does not remove iron bound to transferrin or ferritin, a two-step filtration process was used: filtration with a 100-kD molecular weight cut-off ultracentrifuge filter (Whatman, Clifton, NJ, U.S.A.) was followed by filtration with one having a cut-off at 20 kD. The filtrate was analyzed by direct injection into a reverse-phase liquid chromatography system, using pre-column derivatization with the high-affinity iron chelator CP22 (3-hydroxyl-1-propyl-2-methyl-pyridin-4-one hydrochloride). The system is able to detect iron in a ferric nitrate standard at a concentration as low as 0.02 μM. NPBI brain levels were expressed as micromole per gram of tissue.

Histology. Left hemispheres of all piglets were removed after intracardial perfusion with 4% phosphate-buffered formaldehyde and were processed for light microscopy. The hemispheres were accurately placed into a self-made leaden mall and were cut at fixed intervals into three parts for easier processing for light microscopy. Coronal sections (7 μm) of these brain parts were cut, mounted on a silan-coated glass slide and stained with cresyl violet. The striatum was counted at 17.50 mm in the anterior plane, as shown in a stereotaxic atlas of the pig brain (17). The parietal cortex and hippocampus were both counted at 3.00 mm in the posterior plane (17). Quantification of neuronal viability was performed in the central three lobes of the parietal cortex and in the caput of the caudate nucleus of the striatum using a grid of 100 compartments at 200× magnification. All normal-appearing neurons in the CA 1, CA 2, CA 3, and CA 4 region of the hippocampus and the dentate gyrus were counted. The averaged value was used as the amount of normal-appearing cells in the hippocampus.

Apparently normal neurons were morphologically identified by the presence of typical nuclei with clear nucleoplasm and a distinct nucleolus, surrounded by purple-stained cytoplasm. Neurons were defined as damaged when no distinction could be made between nucleus and cytoplasm (pyknotic or necrotic).

TUNEL labeling. DNA fragmentation was assessed with TUNEL labeling using an *in situ* detection kit (ApopTag peroxidase kit; Intergen, Purchase, NY, U.S.A.). The presence of positively stained nuclei was assessed in cortex, striatum and hippocampus at 200× magnification. A global estimation of TUNEL-positive cells on a three-point scale was made, as described previously (12): a score of 1 (mild) was assigned to piglets with <25% staining of the nuclei, a score of 2 (moderate) to piglets with nuclear staining between 25 and 75%, and a score of 3 (severe) to staining of the nuclei of > 75%.

Statistical analysis. Data are presented as mean ± SEM. Testing for independent samples was performed with the Kruskal-Wallis test. The Wilcoxon signed rank test was used for two dependent samples. Bonferroni correction for multiple testing was performed when appropriate. MRS and MRI data from piglets that died prematurely were left out at the 24-h posthypoxic-ischemic time points, unless the piglets died with a persistently isoelectric amplitude-integrated EEG during the period of secondary energy failure (two vehicle-treated and one allopurinol-treated piglet) (18). Then, the PCr/Pi-ratios were considered to be zero. For histology the brain halves were collected at 24 h or at earlier time points in case of premature death. Ordinal data for TUNEL labeling and nitrotyrosylation were assessed with cross-tabulations using Gamma testing. With an expected variation coefficient of 10% and an expected relative effect of 20%, power analysis revealed that groups of 10 animals each would be sufficient to demonstrate these differences with a β of 0.10 and α of 0.05. A P value <0.05 was considered statistically significant.

RESULTS

Physiologic data. Animal weight, sex, and gestational and postnatal ages were equally distributed among groups. Three

vehicle-, one allopurinol-, and one deferoxamine-treated piglets died between 5 h and 19 h after hypoxia-ischemia due to posthypoxic-ischemic complications such as cardiac failure or gut necrosis (not statistically different). MABP, heart rate, arterial pH, arterial P_{CO_2}, and BE values are shown in Table 1. No significant differences were detected between groups at baseline, the end of hypoxia-ischemia, and at 24 h after hypoxia-ischemia. As expected, a significant increase in heart rate and a decrease in pH and BE was observed in all groups during the hypoxic-ischemic insult compared with baseline values. In vehicle-treated piglets, the heart rate remained significantly increased at 24 h after hypoxia-ischemia, whereas in allopurinol-treated piglets, the BE was significantly increased at 24 h compared with baseline values. There were no relevant changes in arterial P_{CO_2}, glucose, and rectal temperature during the study period for all treatment groups.

Sham-operated piglets did not show differences in MABP, heart rate, arterial pH, P_{CO_2}, and BE levels over time (data not shown).

^{31}P-MRS. Table 2 shows the PCr/Pi ratio, a sensitive indicator for the energy status of the brain tissue, for all treatment groups at baseline, at the end of 1 h of hypoxia-ischemia, and at 24 h after hypoxia-ischemia. During hypoxia-ischemia, there was a rapid, significant decline in PCr/Pi ratios to ≤30% of baseline values in all groups as intended ($p < 0.005$). The decrease of PCr/Pi during the 1-h hypoxic-ischemic insult was similar in the vehicle-, allopurinol-, and deferoxamine-treated groups. Vehicle-treated piglets showed a significant reduction in cerebral energy status at 24 h after hypoxia-ischemia compared with baseline ($p < 0.005$). Preservation of the cerebral energy status was observed in allopurinol- and deferoxamine-treated piglets at 24 h after hypoxia-ischemia compared with baseline. PCr/Pi ratios of sham-operated piglets varied from 1.70 ± 0.31 at baseline to 1.52 ± 0.16 at 24 h (not significantly different).

Table 1. *Physiological data at baseline, at the end of 1 h of hypoxia-ischemia (HI), and at 24 h after HI for vehicle (n = 12), allopurinol (n = 10), and deferoxamine (n = 10) treated piglets*

	Baseline	End of HI	24 h after start of HI
MABP (mm Hg)			
Vehicle	55 ± 4	75 ± 8	58 ± 5†
Allopurinol	57 ± 2	68 ± 8	49 ± 4
Deferoxamine	52 ± 2	65 ± 6	62 ± 4
HR (beats/min)			
Vehicle	160 ± 7	210 ± 14*	194 ± 15*
Allopurinol	168 ± 8	247 ± 12*	189 ± 11†
Deferoxamine	155 ± 7	218 ± 15*	200 ± 13
pH_a			
Vehicle	7.34 ± 0.02	7.11 ± 0.05*	7.43 ± 0.03†
Allopurinol	7.34 ± 0.02	7.22 ± 0.04*	7.48 ± 0.05†
Deferoxamine	7.36 ± 0.02	7.12 ± 0.04*	7.42 ± 0.02†
$Paco_2$ (mm Hg)			
Vehicle	44 ± 2	46 ± 3	39 ± 3
Allopurinol	48 ± 3	40 ± 4	38 ± 5
Deferoxamine	39 ± 2	46 ± 3	39 ± 3
BE (mmol/L)			
Vehicle	−1.7 ± 1.4	−13.6 ± 1.7*	1.7 ± 2.1†
Allopurinol	−0.6 ± 1.3	−10.5 ± 1.4*	4.2 ± 1.0†
Deferoxamine	−3.3 ± 1.3	−15.0 ± 1.8*	0.7 ± 1.0†

Values are mean ± SEM. Wilcoxon signed rank test after Bonferroni correction for multiple testing. HR, heart rate.

* $p < 0.05$ *vs* baseline; † $p < 0.05$ *vs* end of 1 h of hypoxia-ischaemia.

Table 2. *PCr/Pi ratios ratios of vehicle- (n = 12), allopurinol (n = 10), and deferoxamine-treated piglets (n = 10) during baseline, at the end of 1 h of hypoxia-ischaemia (HI) and at 24 h post-HI*

	Baseline	End of HI	24 h after start of HI
Vehicle	2.37 ± 0.22	0.40 ± 0.10**	0.80 ± 0.24**
Allopurinol	2.52 ± 0.22	0.31 ± 0.08**	1.53 ± 0.47
Deferoxamine	3.08 ± 0.42	0.21 ± 0.05**	1.97 ± 0.46†

Values are mean ± SEM. Wilcoxon signed rank test after Bonferroni correction for multiple testing, ** $p < 0.005$ *vs* baseline; † $p < 0.05$ *vs* end of hypoxia-ischemia (HI).

MRI

Water T2 values, which are a sensitive indicator of the possible development of vasogenic edema, are presented in Table 3. Water T2 in the cerebral cortex, striatum, and thalamus was significantly prolonged at 24 h after hypoxia-ischemia compared with baseline in the vehicle-treated, but not in the allopurinol- or deferoxamine-treated piglets. No changes in T2 values were seen in white matter in all treatment groups.

CASPASE-3 ACTIVITY

No significant differences in caspase-3 activity were present between treatment groups at 24 h in cortex, hippocampus, striatum, thalamus, or white matter (Table 4).

ALLOPURINOL AND OXYPURINOL LEVELS IN SERUM

Allopurinol and oxypurinol levels for the allopurinol-treated piglets during the 24-h period of reperfusion are shown in Figure. 1. Allopurinol levels ranged from 22 mg/L directly after the first injection to 6 mg/L at 12 and 24 h after reperfusion. Oxypurinol

Table 3. *Water T2 values (mean ± SEM in ms) of vehicle- (n = 12), allopurinol- (n = 10), and deferoxamine-treated piglets (n = 10) at baseline and at 24 h after hypoxia-ischemia (HI)*

	Baseline	24 h after HI
Cortex		
Vehicle	80 ± 3	93 ± 5*
Allopurinol	93 ± 6	107 ± 12
Deferoxamine	85 ± 5	96 ± 11
Striatum		
Vehicle	72 ± 2	82 ± 3*
Allopurinol	93 ± 10	90 ± 10
Deferoxamine	82 ± 8	83 ± 8
Thalamus		
Vehicle	66 ± 1	68 ± 1*
Allopurinol	84 ± 10	85 ± 9
Deferoxamine	73 ± 6	75 ± 6
White matter		
Vehicle	59 ± 1	62 ± 2
Allopurinol	73 ± 6	71 ± 6
Deferoxamine	64 ± 4	66 ± 6

* $p < .05$ *vs* baseline, Wilcoxon signed rank test.

Table 4. *Caspase-3 activities (pg AMC released/mg protein–min) at 24 h after hypoxia-ischemia in vehicle- (n = 12), allopurinol- (n = 10), and deferoxamine- (n = 10) treated piglets and in sham-operated piglets (n = 5)*

	Cortex	Striatum	Thalamus	White matter
Vehicle	5.0 ± 1.4	5.3 ± 1.7	3.8 ± 3.0	5.7 ± 2.4
Allopurinol	3.1 ± 1.1	2.5 ± 0.9	0.6 ± 0.2	2.4 ± 1.0
Deferoxamine	2.6 ± 1.4	2.2 ± 1.4	1.3 ± 0.7	2.5 ± 1.4
Sham	0.2 ± 0.0	0.3 ± 0.0	0.6 ± 0.1	0.4 ± 0.1

No significant differences between vehicle-, allopurinol-, and deferoxamine-treated piglets were demonstrated. Values are mean ± SEM.

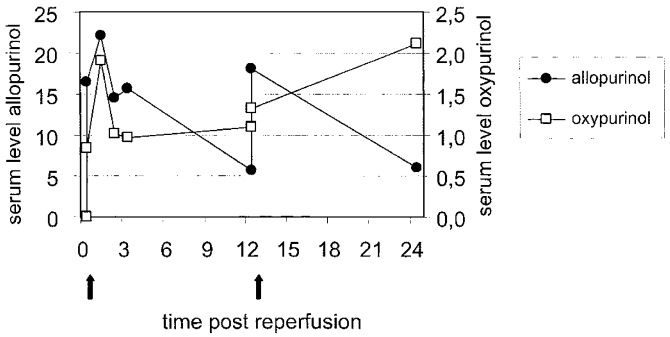

Figure 1. Serum allopurinol and oxypurinol levels. Mean serum allopurinol and oxypurinol levels (mg/L) during the 24 h reperfusion period (n = 5–10) at the different time points. *Arrows* indicate the administration of 20 mg/kg and 10 mg/kg allopurinol at 0 h and 12 h, respectively.

levels ranged from 1.9 mg/L directly after injection of allopurinol and to 2.1 mg/L at 24 h after hypoxia-ischemia.

NPBI LEVELS IN SERUM AND BRAIN

No significant differences in NPBI levels in serum were found between groups at baseline and at 24 h after hypoxia-ischemia (Table 5). NPBI in brain tissue tended to be reduced in the deferoxamine-treated piglets at 24 h after hypoxia-ischemia, but it did not reach a significant difference due to the large SEM.

HISTOLOGY

No differences in amount of normal appearing cells were demonstrated between the treatment groups using cresyl violet staining (Table 6) or TUNEL labeling (Fig. 2).

DISCUSSION

In the present study, beneficial effects of allopurinol and deferoxamine were demonstrated at 24 h after hypoxia-ischemia. Whereas secondary energy failure, *i.e.* a secondary decline in PCr/Pi ratios, was demonstrated in the vehicle-treated piglets compared with baseline levels, no significant

Table 5. *NPBI levels in serum in μmol/L at baseline and at 24 h after hypoxia-ischemia (HI) and in cortical brain tissue (in μmol/g) at 24 h after HI*

Serum	Baseline	24 h	Brain	24 h
Vehicle	2.8 ± 1.6	6.5 ± 4.9		36.2 ± 18.4
Deferoxamine	5.1 ± 3.6	2.9 ± 1.1		8.9 ± 3.6
Sham	0.9 ± 0.7	0.1 ± 0.1		12.0 ± 4.0

Values are mean ± SEM.

Table 6. *Histology: piglets were either treated with vehicle (n = 12), allopurinol (n = 10), deferoxamine (n = 10) or were sham-operated (n = 5)*

Histology	Cortex	Striatum	Hippocampus
Vehicle	155 ± 37	222 ± 60	45 ± 12
Allopurinol	190 ± 59	210 ± 52	60 ± 13
Deferoxamine	207 ± 45	284 ± 62	65 ± 11
Sham	349 ± 71	409 ± 23	101 ± 17

For histology, the number of normal-appearing neurons per field of examination is indicated. No significant differences were demonstrated between the treatment groups. Values are mean ± SEM.

impairment in cerebral energy status was observed in either the allopurinol- and deferoxamine-treated piglets. Simultaneously, increased water T2 values were found only in the gray matter of vehicle-treated piglets. In contrast with these beneficial findings, no significant effects of allopurinol or deferoxamine were demonstrated on caspase-3 activity, histologic outcome, or TUNEL labeling at 24 h after hypoxia-ischemia.

Allopurinol. Peak allopurinol levels were above 20 mg/L in serum and minimal allopurinol levels were above 5 mg/L. The allopurinol serum levels of the piglets in the present study showed a striking similarity to those of asphyxiated human neonates (8). From these levels it can be assumed that allopurinol was adequately dosed to achieve xanthine oxidase inhibition (10). Similar beneficial effects of allopurinol, *i.e.* preservation of brain energy status and reduced brain edema, comparable to those in the present study, have been previously observed in 7-d-old rats, treated *before* onset of hypoxia-ischemia. Neuroprotection in these animals was confirmed with histology at 30 d after hypoxia-ischemia (19, 20). Neuroprotective effects of allopurinol administered *after* the insult have been observed in 7-d-old rats (10), and in newborn lambs (7). Reactive oxygen species–induced brain cell injury can be reduced through inhibition of xanthine oxidase, present in capillary endothelial cells (21), by allopurinol and oxypurinol (22). Other neuroprotective pathways of allopurinol are direct free radical scavenging demonstrated *in vitro* with high concentrations of allopurinol (23), inhibition of neutrophil accumulation (24), chelation of metal ions such as ferric iron (25), and facilitation of electron transport from ferrous iron to ferric cytochrome *c* (26). In contrast with previous observations, no effects on caspase-3 activity, number of apparently normal neurons, or TUNEL labeling were found at 24 h after hypoxia-ischemia. The discrepancy between the reduction of secondary energy failure and brain edema on the one hand, and the lack of histologic neuroprotection on the other hand, might suggest that cell populations in the brain may react differently to allopurinol. Neurons may be less protected than glial cells, whereas these functioning glial cells preserve brain energy status and do not contribute to brain edema. However, recently it has been shown that astroglial cells account for only 14% of the total brain oxygen consumption (27), which makes this suggestion unlikely. A more likely explanation is that administration of allopurinol increases the heterogeneity in the treatment group by rescuing only the moderately damaged piglets, whereas the severely damaged piglets can no longer be rescued. This heterogeneity in the severity of the brain injury

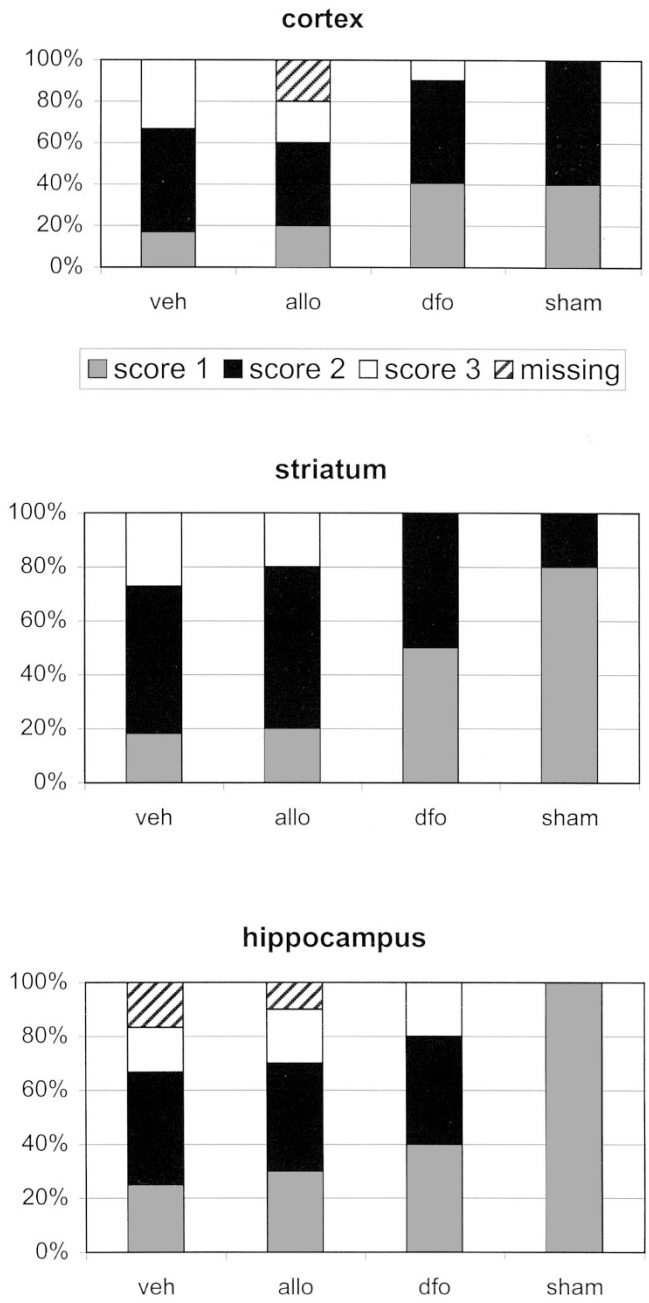

Figure 2. TUNEL score. Percentage of piglets with score 1 (mild, <25% of cells stained), score 2 (moderate, 25–75% of cells stained), or score 3 (severe, >75% of cells stained) on a three-point scale and percentage of piglets with a missing TUNEL labeling are indicated for TUNEL data in cortex, striatum, and hippocampus. No significant differences between groups were demonstrated.

cannot be predicted from ^{31}P-MRS measurements during the insult because all piglets had a similar decrease of PCr/Pi ratios, but rather it reflects individual differences of the piglets.

The increase in heterogeneity with rescue of the moderately damaged piglets only would explain the increase in SEM as seen in the PCr/Pi ratios and water T2 values in cortical areas of allopurinol-treated piglets at 24 h after hypoxia-ischemia (Tables 2 and 3). Furthermore, it is unknown whether the effects of allopurinol on brain edema and brain energy are sustained beyond 24 h after the insult.

Regarding the safety of allopurinol in neonates and the effects of allopurinol on brain energy and edema, and notwithstanding the lack of beneficial effects on histology, we suggest that allopurinol may be used in a combination therapy after perinatal hypoxia-ischemia.

Deferoxamine. In the present study, positive effects of deferoxamine on cerebral energy status and the water T2 values were demonstrated. In previous studies, it was shown that deferoxamine had neuroprotective effects in rats, dogs, and sheep after hypoxia-ischemia, but less so in piglets (7, 11, 28, 29). For deferoxamine to be neuroprotective it must have access to intra- and extracellular iron. Although studies have suggested that deferoxamine is incapable of crossing cell membranes effectively, it does accumulate in the lysosomes and complexes with intralysosomal iron (30). Furthermore, it was shown that deferoxamine can enter the brain of a 7-d-old rat exposed to hypoxia-ischemia (28). A deferoxamine-mediated reduction in brain iron content and lipid peroxidation after hypoxia-ischemia in lambs supports this previous observation (31). NPBI levels in brain tissue tended to be lower in deferoxamine-treated animals as opposed to vehicle-treated animals, but the differences did not reach significance. Measurements of NPBI were performed 12 h after the last dose of deferoxamine. It cannot be excluded that measurements at earlier time points, as have been performed in newborn lambs (31), would have resulted in more reduced levels of NPBI and may be species dependent. Alternatively, it could be argued that the dose of deferoxamine has been too low to obtain reductions of NPBI levels in the brain. The present dose of deferoxamine treatment was chosen, because deLemos *et al.* (32) described toxic effects of high-dose deferoxamine treatment on the cardiovascular system in baboons. Earlier studies in newborn lambs showed that even low-dose deferoxamine (2.5 mg/kg) led to a reduction in NPBI in the brain at 3 h after hypoxia-ischemia (31). With the present dose of 12.5 mg/kg/d deferoxamine, we did not detect any negative effects.

Several mechanisms of action for deferoxamine treatment have been proposed. Firstly, deferoxamine binds ferric iron and thereby prevents the formation of the very potent hydroxyl radical *via* the Fenton/Haber-Weiss reaction (4). Secondly, deferoxamine can scavenge superoxide and hydroxyl radicals in concentrations of 0.5–1 mM (28) and prevents neutrophil-mediated killing of endothelial cells in cell cultures, which might reduce the inflammatory response after hypoxia-ischemia and reperfusion (33). Previous studies in newborn lambs showed that rescue treatment with deferoxamine increased brain perfusion, whereas oxygen metabolism, electrocortical brain activity, and cortical neuronal cell membrane Na^+, K^+-ATP-ase activity were stable in the first 3.5 h after reperfusion and were comparable to baseline values (7, 34). In a clinical study in 50 severely asphyxiated full-term infants, children with severe birth asphyxia and an adverse outcome at 2 y of age had higher levels of NPBI in plasma during the first 8 h of life, which suggests a neurotoxic role of NPBI (35).

Again, as with allopurinol, deferoxamine resulted in preservation of cerebral energy status and preserved water T2 properties, indicating reduced vasogenic edema without effects on histologic outcome. Effects of deferoxamine may be different on glial cells and neurons.

It is unknown whether brain edema at 24 h after hypoxia-ischemia is a good predictor of long-term outcome. In view of this point, the observation that the MR-measured extent of cerebral edema 2 d after the insult in hypoxic-ischemic mice correlated with long-term histologic outcome is of interest (36).

In the present study, piglets were observed for 24 h after hypoxia-ischemia. Because Lorek *et al.* (6) reported that the secondary energy failure starts as early as 12 h after hypoxia-ischemia, and no significant differences in PCr/Pi ratios were demonstrated between 24 and 48 h after hypoxia-ischemia, the time point of 24 h was chosen to establish "early outcome." Therefore, in the present and in a previous study (6), a 24-h observation period after hypoxia-ischemia was considered a valid approach to examine the development of brain injury. It could be argued that, in the present study, the period of secondary energy failure was only postponed after the first 24 h after hypoxia-ischemia. It needs to be stressed that definite conclusions can only be drawn from long-term follow-up studies.

CONCLUSION

In summary, the present study demonstrated that allopurinol and deferoxamine maintained cerebral energy status after global hypoxia-ischemia in newborn piglets, however, no effects on neurologic cell death at 24 h after the insult became apparent. Future studies are necessary to assess the long-term histologic outcome as well as the potential of these drugs in combination with other neuroprotective strategies.

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