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Research Report

Neuroprotective effects of topiramate after hypoxia–ischemia in newborn piglets

Susanne Schubert[a], Ulrich Brandl[a], Michael Brodhun[b], Christiane Ulrich[a], Jens Spaltmann[a], Nico Fiedler[a], Reinhard Bauer[c,*]

[a]Department of Neuropediatrics, Children Hospital, Universitätsklinikum Jena, Friedrich Schiller University, D-07740 Jena, Germany
[b]Department of Pathology, Universitätsklinikum Jena, Friedrich Schiller University, D-07740 Jena, Germany
[c]Department of Pathophysiology and Pathobiochemistry, Universitätsklinikum Jena, Friedrich Schiller University, D-07740 Jena, Germany

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Abstract

Background: Perinatal hypoxia–ischemia (HI) is associated with delayed cerebral damage, which involves receptor-mediated excitotoxicity. Until now, successful interventions to reduce excitotoxicity early after HI in experimental settings failed to transform into clinical applications owing to negative side effects. A promising new approach using the anticonvulsant Topiramate (TPM) has shown to be effective to reduce brain damage after early HI in a rodent model of combined TPM-hypothermia. Here, we used TPM solely administered 1 h after HI in a neonatal piglet model in order to verify possible neuroprotection. *Methods:* Newborn piglets were subjected to HI by transient occlusion of carotid arteries and hypotension (62–65% of baseline). Fifteen minutes later, an additional reduction of the inspired oxygen fraction to 0.06 was performed for 13 min. One cohort (VEHICLE, $n = 8$) received saline solution i.v. 1 h after HI and then twice a day. Two further cohorts were treated at same times with TPM (HI-TPM10, $n = 8$, loading dose 20 mg/kg; maintenance dose 10 mg/kg/day; HI-TPM20, $n = 8$, loading dose 50 mg/kg; maintenance dose 20 mg/kg/day). Untreated animals (CONTROL, $n = 8$) received all experimental procedures except HI. Animals were monitored 3 days after HI concerning occurrence of seizures as well as neurological and behavioral functions. After 72 h, the brains were perfused and processed to assess neuronal loss and DNA-fragments (TUNEL staining). *Results:* There was a significant reduction of neuronal cell loss in HI-TPM20 animals. However, apoptosis was increased in the frontal white matter of HI-TPM20 animals. *Conclusions:* Exclusive TPM treatment shows neuroprotection in newborn piglets after HI.

Theme: Disorders of the nervous system
Topic: Ischemia

Keywords: Hypoxia–Ischemia; Newborn; Neuroprotection; Piglet; Topiramate

1. Introduction

Perinatal hypoxia–ischemia (HI) is the single most important cause of brain injury in the newborn, and has consequences that are potentially devastating and lifelong [5]. HI leads to different neuropathological manifestations, depending on the maturity of the newborn. It has been proposed that neurons connected in already established neuronal circuits appear to be especially vulnerable to excitotoxic damage based on a hyperactivity of the major excitatory glutamatergic input [12]. Therefore, predominant brain damage is seen in the parasagittal region of the cerebral cortex, the basal ganglia, and the hippocampus [21]. The principal mechanism of early brain damage is initiated by energy depletion, which led to depolarization, excessive extracellular glutamate release, and hence prolonged activation of glutamate receptors. Subsequently, intracellular calcium accumulation results were induced predominantly by voltage- and ligand-dependent calcium

* Corresponding author. Fax: +49 3641 938954.
E-mail address: Reinhard.Bauer@mti.uni-jena.de (R. Bauer).

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channels. This activates a variety of calcium-mediated deleterious events leading to secondary energy failure and triggering cellular apoptosis [12].

Until now, concepts of pharmacotherapeutic treatment of early brain damage after HI were focused on single aspects of intervention and remained ineffective, presumably due to the complexity of the ongoing damaging processes. Increasing evidence appears that clinical neuroprotection is more likely to be effective if multiple strategies are employed to interrupt simultaneously several different pathways that promote intracellular calcium accumulation [4]. Recently, it has been shown that early administration of Topiramate (TPM), a neuroprotective agent, in combination with later-onset cooling effectively reduces HI brain injury in a neonatal rat stroke model [15].

However, TPM alone appears to fulfill requirement of multiple interaction with potential cell-injuring pathways: TPM is a structurally novel broad spectrum antiepileptic drug (AED) with several mechanisms of action. These include a negative modulatory effect (use-dependent blockade) on presynaptic voltage-activated neuronal sodium channels. Electrophysiological studies have demonstrated that TPM suppresses the presynaptic voltage-sensitive sodium channel of excitatory synapses, which may reduce excessive glutamate release after HI impact [19]. Furthermore, TMP enhances GABA-mediated chloride flux and GABA-evoked chloride currents in murine brain neurons and increases seizure threshold [22]. It appears possible that TMP induces neuroprotection during HI by GABAergic hyperpolarization in the term neonate. Interestingly, TPM has been shown to attenuate AMPA-kainate receptor-mediated cell death and calcium influx, as well as kainate-evoked currents in developing oligodendrocytes. Therefore, a protective role for TPM was demonstrated in a rat pup model of periventricular leukomalacia [6]. Finally, a negative modulatory effect on some L-type high-voltage-activated calcium channels, and inhibition of the carbonic anhydrase isozymes CA-II and CA IV, has been reported for TPM [18].

Brain maturation in newborn piglets is similar to that in human infants at birth. Therefore, we have developed a model of infant human hypoxia/ischemia using term newborn piglets. We hypothesized that early administration of TPM may decrease seizure activity, improve neurological outcome, and reduce brain damage in newborn piglets subjected to HI.

2. Materials and methods

2.1. Animals

All surgical and experimental procedures were approved by the committee of the Thuringian State Government on Animal Research. Piglets (n = 32; aged 2 to 5 days old, body weight 1984 g ± 233 g) were randomly assigned. Untreated animals (CONTROL, n = 8) received all experimental procedures except HI. Remaining animals were subjected to bilateral carotid artery occlusion and arterial hypotension. One cohort (VEHICLE, n = 8) was submitted to hypoxia–ischemia and received intravenous saline solution 0.9% 1 h after the insult and twice a day over the following 2 days. A further cohort (HI-TPM10, n = 8) was treated with an intravenous loading dose of soluted TPM 20 mg/kg 1 h after hypoxia–ischemia. The TPM maintenance dose was 10 mg/kg/day in two dosages on the following 2 days. The remaining cohort (HI-TPM20, n = 8) received TPM with a loading dose of 50 mg/kg and a maintenance dose of 20 mg/kg/day in two dosages per day on the following 2 days. The respective volume to administer “loading dose” of the vehicle/TPM was 2 ml/kg and 0.5 ml/kg to administer “maintenance dose”. Treatment was performed in a blinded fashion.

2.2. Anesthesia and surgical preparation

The piglets were anesthetized with 0.8% isoflurane in 70% nitrous oxide and 30% oxygen by mask. A catheter was inserted into a superficial ear vein for drug administration. Animals were intubated, immobilized with pancuronium bromide (0.2 mg/kg body weight/h, i.v.), and artificially ventilated (Servo Ventilator 900 C, Siemens-Elema, Sweden). Ventilation was controlled by continuous endexpiratory CO_2 monitoring and half-hour arterial blood gas checks. Inflatable occluders were positioned around both common carotid arteries. Polyurethane catheters were introduced through both umbilical arteries into the abdominal aorta. Opposite endings were advanced subcutaneously to the left flank and fixed after removal through a small skin incision. Then, four screw electrodes were drilled into the skull for unipolar electrocorticogram (ECoG) recordings, which were stored for off-line data analysis (Fast Fourier Transformation).

2.3. Experimental protocol

2.3.1. HI insult administration

After CONTROL values had been obtained, piglets of the groups VEHICLE, HI-TPM10 and HI-TPM20 underwent a cerebral hypoxic–ischemic insult. First, common carotid arteries were bilaterally occluded and blood was withdrawn from the arterial line into a heparinized syringe to reduce ABP to about 40 mm Hg (~40 mm Hg, 62–65% of baseline). The amount of arterial hypotension was maintained over the given amount of time for hypotension by continuous ABP titration (e.g., appropriate blood infusion/withdrawal). Fifteen minutes later, FiO_2 was reduced from 0.35 to 0.06 for another 13 min. Successful hypoxia–ischemia was defined when ECoG activity was reduced more than 85% compared to baseline (Fig. 1). Five animals were excluded because of insufficient reduction of

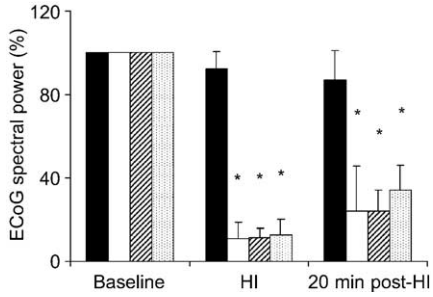

Fig. 1. Effect of hypoxic–ischemic impact (HI), on ECoG activity in newborn piglets. Note that similar reduction occurred in total spectral power during the late period of HI (estimated between 5th and 13th minute of severe hypoxia) with subsequent gradual recovery 20 min after ending the HI insult (20 min post-HI) in all three groups underwent the same procedure of HI (VEHICLE, $n = 8$, blank columns; group TPM-10, $n = 8$, hatched columns; group TPM-20, $n = 8$, dotted columns), compared with untreated animals (CONTROL, $n = 8$, filled columns). Values are presented as means ± SD. *Result significantly different to CONTROL ($P < 0.05$).

ECoG activity. The recovery period was started by deflating the vessel, restoring blood flow, and administering pure oxygen for 30 min. Then, animals were weaned from artificial ventilation.

The blood pH, PCO_2, and PO_2 were measured with a blood gas analyzer (model ABL50, Radiometer, Copenhagen, Denmark). Blood hemoglobin and oxygen saturation were measured using a hemoximeter (model OSM3, Radiometer, Copenhagen, Denmark), and the blood glucose and lactate contents were measured with an electrolyte, metabolite laboratory EML105® (Radiometer, Copenhagen, Denmark) and corrected to the body temperature of the animal at the time of sampling.

2.3.2. Monitoring period

One hour after the HI insult, HI-TPM10 and HI-TPM20 groups received loading doses, whereas the VEHICLE and CONTROL groups received the same volume of saline solution using the arterial line. Animals were fed with artificial piglet formula milk every 6 h. Spontaneous behavior and occurrence of seizures were recorded by video monitoring 15 h per day. Furthermore, the animals underwent measurements of physiological parameters and neurological examination twice a day and ECoG recordings four times a day. A pediatric neurologist blinded to the experimental performance performed neurological examination. Results were recorded and scored from 10 to 36 with 36 being normal according to an adapted standardized scoring approach for piglets [1] (Table 1). Neurologic function in piglets was quantified based on five different components: (1) vigilance with the subitems "mental status" (level of consciousness: coma = 0, stupor = 1, lethargy = 3, awake = 4) and "behavior" (defense on manipulation, contact to other piglets, vocalization, motivation to explore the environment: no = 0, weak = 1, aggressive = 3, normal = 4); (2) cranial nerves with the subitems "pupils" (light reaction and corneal reflex: unreactive = 1, sluggish, side difference = 2, normal = 3) and "oculo-vestibular reflex" (absent = 1, nystagmus = 2, present = 3); (3) reflexes with the subitems "stepping" [evaluation of extensor postural thrust and wheelbarrowing (walking on forelimbs with hind limbs elevated): absent = 1, present either forelimb or posterior = 2, possible allover = 3] and "righting" (possibility to move or roll over from the backside: absent = 1, present = 2); (4) motor with the subitems "tonus" (muscle tone in trunk and limbs: atonic or hypertonic all over = 1, partly atonic = 2 or hypertonic = 3, normal = 4) and "standing" (not possible = 1, paretic = 2, bears weight unsure = 3, normal = 4); and (5) coordination with the subitems "walking" (not possible = 1, creeping/paretic = 2, walks but falls = 3, normal = 4) and "feeding behavior" [no swallowing reflex = 1, has to be fed = 2, no appetite = 3, fasting (e.g., diarrhea, vomiting) = 4, normal = 5].

2.3.3. Neuropathologic evaluation

After about 68 h, the piglets were re-anesthetized and brains were perfused. All brains were fixed in paraffin; 5-μm sections were stained with hematoxylin and eosin for semiquantative analysis of extent and severity of injury in cerebral cortex, hippocampus, basal ganglia, thalamus, cerebellum, and white matter tracts [3], with scores defined by the percentage of damaged cells (1 ≤ 10%, 2 = 11–30%, 3 = 31–50%, and 4 > 50%). Scoring was performed by a neuropathologist (M.B.) blinded to the experimental group. Apoptotic activity was detected by terminale desnucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) as described previously [2].

2.4. Statistical analysis

Data are reported as means ± SD, if not otherwise indicated. Initial comparison was done for physiological parameters studied using two-way ANOVA with one factor "treatment", which considered possible effects of HI as well as TPM administration. The second factor "stages" considered repeated measures along the experimental

Table 1
Standardized scoring approach for neurological examination of newborn piglets according to [1]

Neurological item	Subitem	Score
Vigilance	Mental status	1–4
	Behavior	1–4
Cranial nerves	Pupils	1–3
	Oculo-vestibular reflex	1–3
Reflexes	Stepping	1–3
	Righting	1–2
Motor	Tonus	1–4
	Standing	1–4
Coordination	Walking	1–4
	Feeding behavior	1–5
	Total	10–36

approach. Post hoc comparisons between groups were made with multiple *t* tests for all pair-wise multiple comparisons. A Bonferroni adjustment was performed to evaluate significant differences. Comparisons of measurements between baseline and different measures along the experimental approach within the groups were made with one-way ANOVA, with repeated measures. Post hoc comparisons were made with Dunnett's test for multiple comparisons versus baseline. Comparisons of neuroscore and neuropathologic data were performed by Kruskal–Wallis one-way ANOVA on ranks. Post hoc comparisons were made with Dunn's test for multiple comparisons versus baseline. Differences were considered significant when $P < 0.05$.

3. Results

For piglets subjected to HI, all groups exhibited a similar degree of metabolic acidosis, as indicated by reduced pH and marked increase of arterial lactate content ($P < 0.05$, Table 2). Furthermore, concurrent alterations in electrical brain activity and hyperglycemia occurred to a similar degree in each of these groups (Fig. 1, Table 2, $P < 0.05$).

All animals in the CONTROL and TPM-treated groups survived during the 72-h observation period. Two animals of the VEHICLE group died (9.5 h and 41.5 h after HI). Autopsy showed brain stem herniation as a result of brain edema.

Table 2
Effect of hypoxic–ischemic insult and post-insult TPM treatment on arterial blood gases, acid–base balance, and metabolic parameters in newborn piglets

	Baseline	HI	Recovery 30 min	Recovery 20 h	Recovery 68 h
Arterial blood pressure (mm Hg)					
CONTROL	59 ± 8	61 ± 8	69 ± 5	70 ± 10	66 ± 8
Vehicle	65 ± 7	41 ± 3*	84 ± 18	69 ± 10	72 ± 8
HI-TPM10	63 ± 8	41 ± 2*	87 ± 16	64 ± 5	74 ± 7
HI-TPM20	66 ± 4	41 ± 2*	81 ± 11	67 ± 7	74 ± 5
Rectal temperature (°C)					
CONTROL	38.0 ± 0.2	38.0 ± 0.2	38.0 ± 0.2	38.5 ± 0.5	38.1 ± 0.3
Vehicle	37.9 ± 0.5	37.8 ± 0.4	38.0 ± 0.3	38.4 ± 0.2	37.9 ± 0.7
HI-TPM10	38.0 ± 0.4	38.0 ± 0.2	38.1 ± 0.1	38.1 ± 0.3	37.8 ± 0.3
HI-TPM20	38.2 ± 0.1	38.0 ± 0.2	38.1 ± 0.3	38.4 ± 0.4	38.1 ± 0.5
Arterial PCO_2 (mm Hg)					
CONTROL	40 ± 2	40 ± 4	42 ± 6	37 ± 4	38 ± 4
Vehicle	40 ± 5	42 ± 10	38 ± 6	33 ± 2	31 ± 6
HI-TPM10	38 ± 4	38 ± 10	44 ± 10	31 ± 4	41 ± 3
HI-TPM20	39 ± 3	33 ± 5	36 ± 7	33 ± 7	36 ± 4
Arterial pH					
CONTROL	7.46 ± 0.02	7.46 ± 0.04	7.46 ± 0.03	7.43 ± 0.07	7.40 ± 0.05
Vehicle	7.45 ± 0.04	7.25 ± 0.10*	7.38 ± 0.08	7.47 ± 0.03	7.45 ± 0.00
HI-TPM10	7.45 ± 0.04	7.23 ± 0.07*	7.32 ± 0.10*	7.41 ± 0.10	7.33 ± 0.02
HI-TPM20	7.48 ± 0.05	7.34 ± 0.08*	7.39 ± 0.03*	7.44 ± 0.12	7.40 ± 0.04
Arterial PO_2 (mm Hg)					
CONTROL	131 ± 39	121 ± 29	90 ± 25	84 ± 10	135 ± 52
Vehicle	121 ± 20	19 ± 2*	129 ± 49	85 ± 9	98 ± 9
HI-TPM10	134 ± 33	20 ± 3*	132 ± 28	89 ± 14	170 ± 34
HI-TPM20	141 ± 30	20 ± 2*	140 ± 25	99 ± 9*	170 ± 44
Arterial glucose (mmol/l)					
CONTROL	6.7 ± 1.2	6.6 ± 1.2	7.1 ± 1.9	6.0 ± 1.0	5.1 ± 0.5
Vehicle	6.1 ± 2.0	12.8 ± 4.8*	11.8 ± 6.6	6.2 ± 1.5	5.5 ± 0.5
HI-TPM10	5.7 ± 0.6	11.5 ± 3.2*	11.5 ± 3.6	5.7 ± 1.3	5.1 ± 1.1
HI-TPM20	5.8 ± 0.8	10.2 ± 2.8*	10.5 ± 3.2	6.3 ± 1.4	5.2 ± 0.4
Arterial lactate (mmol/l)					
CONTROL	2.9 ± 0.8	2.6 ± 1.1	2.5 ± 0.7	3.8 ± 2.1	2.7 ± 2.1
Vehicle	2.3 ± 0.8	10.8 ± 1.9*	7.1 ± 1.8*	4.2 ± 2.6	2.1 ± 0.8
HI-TPM10	2.2 ± 0.7	9.3 ± 4.4*	5.9 ± 1.6*	3.5 ± 3.1	1.9 ± 1.1
HI-TPM20	2.6 ± 0.6	10.7 ± 4.6*	7.1 ± 1.5*	3.3 ± 3.6	1.8 ± 0.4

Values are presented as means ± SD.

* $P < 0.05$. *Significant differences between untreated control group (CONTROL) and groups which underwent hypoxic–ischemic insult without post-HI treatment (Vehicle) or with post-HI treatment with moderate TPM dosage (HI-TPM10, i.v. loading dose 20 mg/kg 1 h after HI, maintenance dose 10 mg/kg/day) as well as high TPM dosage (HI-TPM20, i.v. loading dose 50 mg/kg 1 h after HI, maintenance dose 20 mg/kg/day).

No seizures occurred in the CONTROL group. In the HI-VEHICLE group, 7 of 8 piglets had seizures during the first night (88%), 4 of 7 during the second (57%), and 4 of 6 (67%) during the third day post-HI. In the HI-TPM10 group, seizures occurred in 5 piglets during the first night (63%), and 3 (38%) and 4 (50%) during the next 2 days. In the HI-TPM20 group, four animals (50%) exhibited seizures during the first night, and two animals each of the next 2 days (25%). Multiple comparison of seizure frequency did not reveal significant differences between animal groups of different treatment procedures post-HI.

Estimation of spontaneous behavior and sensory–motor response revealed that no relevant effects on vigilance occurred throughout the observation period in all groups studied. However, HI provoked sustained neurological deficits 1 to 3 days after injury for all animal groups subjected to HI (Fig. 2, $P < 0.05$).

Histological examination revealed that the frontal, parietotemporal temporoparietal, and occipital cortices, as well as striatum and hippocampus, were markedly injured by HI. Cell destruction was most pronounced in the VEHICLE group (Fig. 3, $P < 0.05$). A significant increase of hypoxic–ischemic neuronal damage was also verified in the temporoparietal cortex in HI-TPM10 animals (Fig. 3, $P < 0.05$). In contrast, HI-TPM20 animals exhibited a markedly reduced amount of neuronal damage compared with VEHICLE-treated and even HI-TPM10 animals (Fig. 3, $P < 0.05$). An increased incidence of TUNEL positive cells was detected solely in the frontal white matter of HI-TPM20 animals in comparison with the untreated animals (Fig. 4, $P < 0.05$).

4. Discussion

Our results demonstrate that TPM can significantly reduce neuronal cell loss after severe HI insult. This effect was dose-dependent. No significant side effects related to vigilance, or neurological and feeding behavior were observed at the doses used in this study. Consequently, body weight development was similar throughout the observation period in all groups investigated. These results indicate that TPM is well tolerated at neuroprotective doses. Furthermore, neurological deficits appeared to be less severe 20 h after HI in TPM-treated animals compared to placebo-treated animals. This was more prominent at the high dose of TPM.

Based on a general goal to achieve an application for potential clinical use, an appropriate range of effective doses has been used. The dosages used in this experimental application represent the dose range known to be effective antiepileptically in human infants [9], but respective data from newborn piglets are lacking. However, it has been shown that the pharmacokinetics of TPM appears to be quite similar as known from human babies with comparable mean plasma clearance and terminal half-life [8,9]. Furthermore, similar TPM doses were administered intravenously in newborn piglets and were well tolerated, with minimal effects on hemodynamics or neurophysiologic scores [8].Onset, type and duration of drug administration were performed in order to verify predetermined effective doses from 1 h after insult up to the third day after insult. Time window for TPM medication was chosen in order to perform tentative neuroprotective therapy throughout the whole period of cerebral threat, i.e., inclusive subsequent

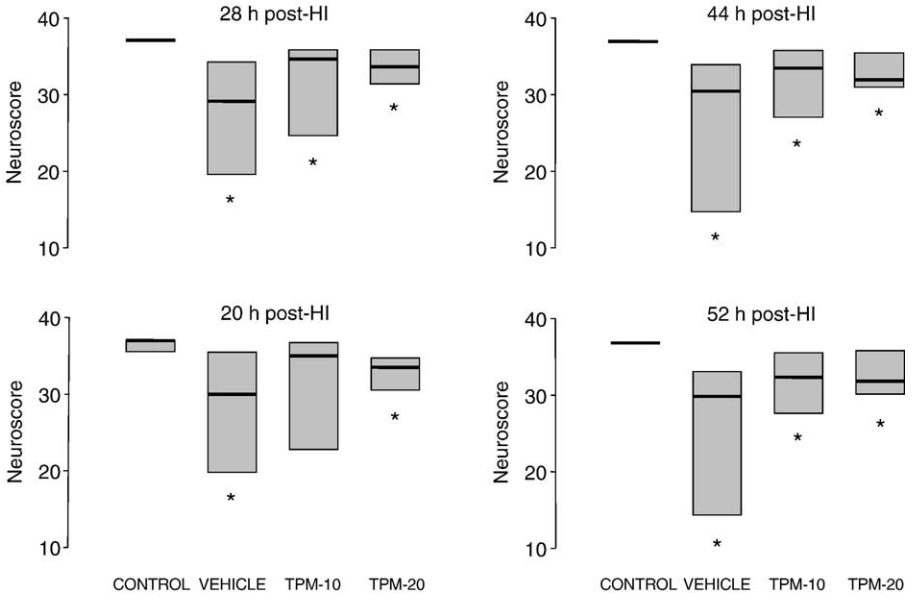

Fig. 2. Box plot of HI and TPM treatment effects on neurologic functions and behavioral skills observed 20, 28, 44, and 52 h after HI and quantified by an adapted standardized scoring approach for piglets [1]. (CONTROL, $n = 8$; VEHICLE, $n = 8$; group TPM-10, $n = 8$; group TPM-20, $n = 8$). $P < 0.05$, *comparison with untreated control animals.

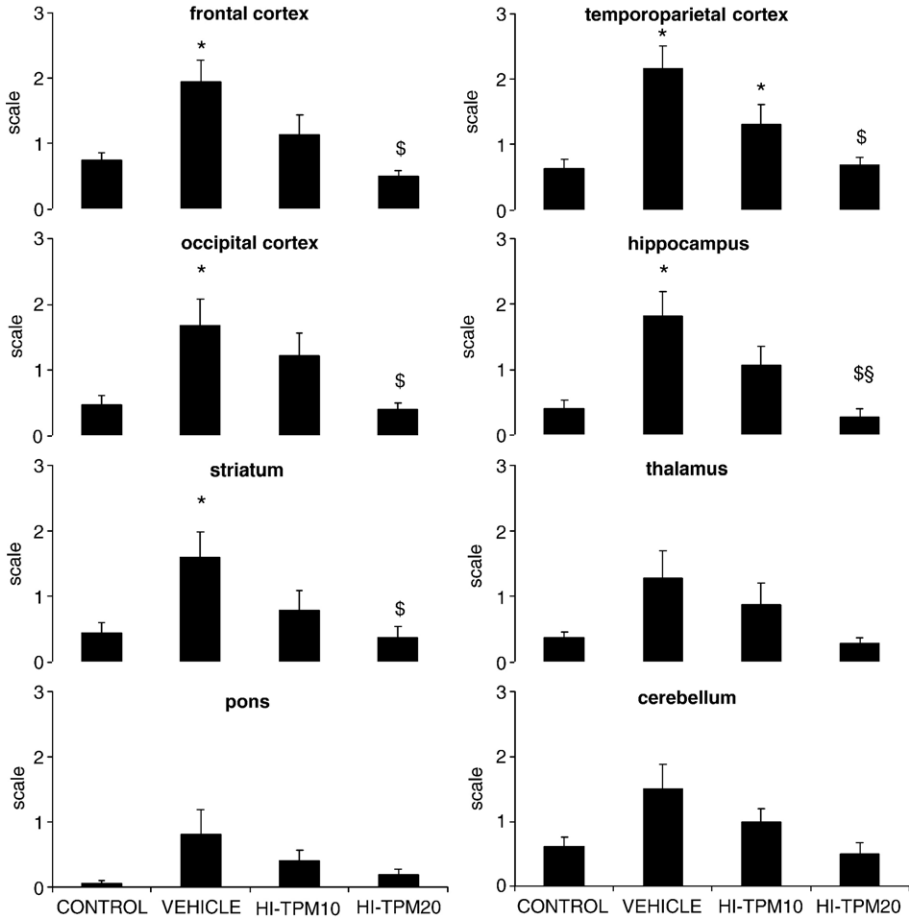

Fig. 3. Estimation of hypoxic–ischemic brain damage by semiquantative analysis on sections stained with hematoxylin and eosin graded on a scale of 0 to 4. (CONTROL, $n = 8$; VEHICLE, $n = 8$; group TPM-10, $n = 8$; group TPM-20, $n = 8$). Values are presented as means ± SD. $^{*,\$,\S}P < 0.05$, *comparison with CONTROL, $^{\$}$comparison with VEHICLE, §comparison with TPM10.

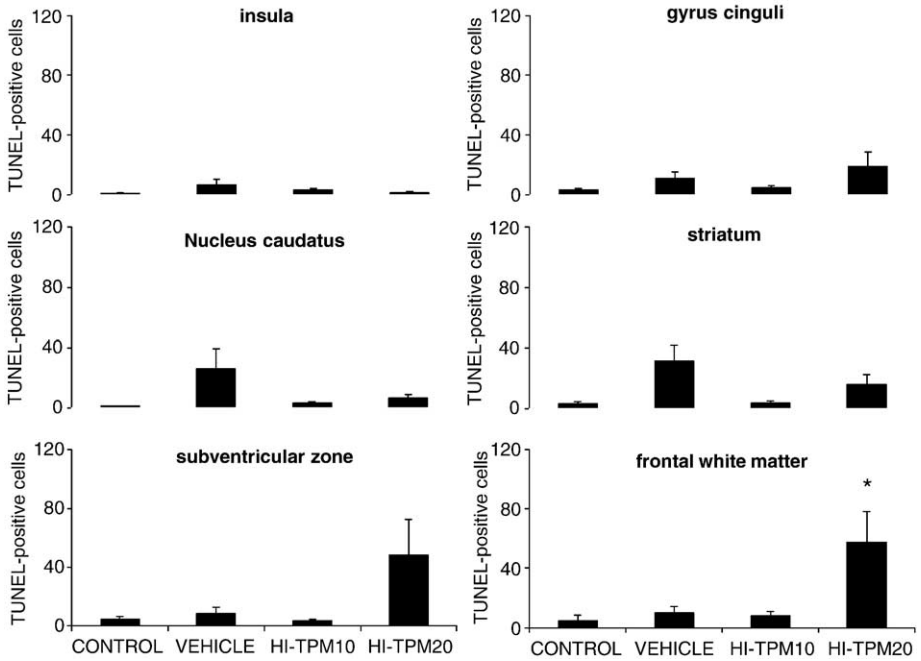

Fig. 4. Estimation of apoptotic activity by nick-end labeling (TUNEL) cells (CONTROL, $n = 8$; VEHICLE, $n = 8$; group TPM-10, $n = 8$; group TPM-20, $n = 8$). Values are presented as means ± SD. $^{*}P < 0.05$, *comparison with CONTROL.

periods of delayed ("secondary") cerebral energy failure after acute hypoxia–ischemia, as already shown in the newborn piglet [16].

Severity of perinatal HI impact depends on intensity and duration. We used a survival model of HI in newborn piglets, which was prone to induce moderate to severe neuronal damage in the forebrain, with life-threatening complications. Two of eight animals of the VEHICLE-treated group died after brain edema developed, as suggested by macroscopic signs at necropsy. Brain swelling after life-threatening perinatal HI is induced by intracellular water accumulation due to EAA-mediated intracellular sodium overload (cytotoxic brain edema) and/or intercellular space enlargement due to cerebrovascular injury and extravasation of protein-rich fluids (vasogenic brain edema). However, our experimental approach did not afford a clarification of underlying mechanisms responsible for secondary brain swelling. Furthermore, a possible impact of disturbed cellular volume regulation on neuronal cell death in the newborn brain remains doubtful [20]. Nevertheless, the herein used model produces a consistent level of neuronal damage and appears useful for examining potential neuroprotective therapies in the neonatal piglet brain.

The primary effect of neuronal injury due to HI is known to be widespread depolarization with profound disturbance of cell-membrane function including the highly regulated transmembraneous ionic fluxes. Extent of local O_2 deprivation determines the fate of compromised neuronal cells. We assume that experimental conditions used herein prevented widespread terminal depolarization caused by disruption of energy production, resulting in a transient breakdown of transmembraneous ionic gradients of all cellular structures involved and subsequent necrosis. This is inferred from time-dependent response of ECoG, which was markedly depressed (Fig. 1), but only seldom isoelectric for a short time period immediately on end of HI. Hence, secondary mechanisms of neuronal injury appear to be mainly responsible for neuronal cell loss. It appears possible that the neuroprotective effect of TPM is linked to influencing the amount of HI-induced excessive glutamate release, because TPM is able to suppress presynaptic voltage-sensitive sodium channel of excitatory synapses after HI impact [19]. However, a proposed stabilization of electric membrane functioning by TMP was diminished after HI because seizure frequency was not significantly reduced in the TPM-treated groups. This occurred even in the high-dose group. The anticonvulsant potency of TPM in the newborn brain after HI brain damage is not well evaluated yet. Koh et al. (2001) reported a blockade of hypoxia-induced seizures in newborn rat pups with prehypoxic TPM administration [13]. Furthermore, TPM's anticonvulsant efficacy decreased with increasing doses. We consider that the study design used is not appropriate to verify dose-dependent anticonvulsant efficacy of TPM on HI-induced seizures in the newborn piglet brain. Nevertheless, results presented herein indicate a neuroprotective potential of TPM, which is obviously not mainly caused by its anticonvulsive effect.

An effective neuroprotection was also reported in a neonatal rat pup model of severe HI by combined early TPM administration and late-onset mild hypothermia [15]. In contrast, neither TPM nor delayed hypothermia alone conferred protection in this study. Different findings between the report on newborn piglets herein and the referred results in rat pups after HI follow obviously from different protocols in dosage and administration. Rat pups received a single TPM dose 30 mg/kg early after HI. Even if interspecies differences of TPM dosage between newborn rat and piglet are not evaluated in terms of neuroprotective as well as antiepileptic effects, we assume that the main reason of failed neuroprotection of TPM in rat pups after HI resulted from underdosage.

Neurological and behavioral deficits quantified by the neuroscore showed a tendency of milder occurrence in the TPM-treated groups. However, the neuroscore differences did not reach the significance level between the groups which suffered from HI. In contrast, comparison with the untreated CONTROL group was mostly different, as indicated in Fig. 2. We consider the amount of the HI-induced brain injury verified by histology to be moderate in vehicle-treated animals and rather mild to moderate (dose-dependent) in TPM-treated ones. Indeed, we did not observe any regions with mass necrosis and neuropathologic evaluation based on quantification of selective neuronal cell damage, accordingly. Therefore, we have to consider that the variability in the amount of neurological and behavioral deficits due to a narrower range of moderate to mild neuropathologic injury was unable to reflect treatment effects. This may reflect an improved ability of compensation in injured newborn piglets with regard to the rather basic neurological and behavioral functions assessed. Similar findings of evaluating significant neuropathological differences but at least tendencies in altered neurology scores were reported by comparable experimental approaches [7,14].

A neuropathological result needs particular mention. An increased rate of cell damage labeled by TUNEL staining appeared after high-dose TPM administration and was predominantly located in white matter structures. We believe that this finding may indicate a complex drug interaction which is presumably responsible for programmed cell death activation during myelination [11,17]. Furthermore, it confirms results from rat pups which underwent dose-dependent TPM administration with increasing rates of apoptosis at single doses >50 mg/kg [10]. However, these results do not substantiate long-term consequences, which have to be proven in subsequent studies.

In summary, these results demonstrate neuroprotective efficacy of the clinically available drug TPM after HI in newborn piglets. We suggest that multiple mechanisms of voltage- and ligand-dependent membrane potential stabilization may act synergistically. The results need confirmation

by long-term validation before clinical application can be considered.

Acknowledgments

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