Influence of Allopurinol on Evoked Cortical Afterdischarges During Early Ontogenesis

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Summary

The aim of our study was to test the hypothesis, whether repeated allopurinol pre-treatment (in dose of 135 mg/kg s.c.) can influence changes of brain excitability caused by long-term hypoxia exposition in young immature rats. Rat pups were exposed together with their mother in to an intermittent hypobaric hypoxia (simulated altitude of 7 000 m) since the day of birth till the 11th day (youngest experimental group) or 17th day for 8 hours a day. Allopurinol was administered daily immediately before each hypoxia exposition. The duration of evoked afterdischarges (ADs) and the shape of evoked graphoelements were evaluated in 12, 18, 25 and 35-day-old freely moving male pups. Hypobaric hypoxia prolonged the duration of ADs in 12, 18 and 25-day-old rats. The ADs were prolonged in 35-day-old rats only after the first stimulation. Allopurinol shorted the duration of ADs only in 12-day-old pups. In older experimental group the effect of allopurinol treatment was less pronounced.

Key words

Allopurinol • Hypoxia • Evoked potentials • Development • Rat

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Introduction

Prenatal and perinatal hypoxic-ischemic injury is one of the major health risk. It induces damage of neuronal circuitry, neurodegeneration and disbalance of excitatory and inhibitory neurotransmitters (Nyakas *et al.* 1996).

Glutamate release plays a critical role in neuronal cell death after cerebral hypoxia/ischemia. In neonatal rats, it was shown that glutamate release during and after hypoxic–ischemic insult could evoke epileptogenic activity and that this effect was dependent on the maturity of the brain. In rats, the most marked effect was observed 10 to 12 days after the birth (Berger and Garnier 1999).

The reoxygenation/reperfusion of previously hypoxic-ischemic brain is essential to recover brain functions. However, reoxygenation/reperfusion paradoxically leads to the formation of highly reactive oxygen free radicals and increases mortality and morbidity (Cowan *et al.* 2003). Restoration of blood and oxygen supply to the brain may give rise to activation of multiple pathways (metabolization of arachidonic acid, activation of glutamate receptors increase in intracellular calcium, activation of proteases and endonucleases, etc.). During this processes, reactive oxygen species (ROS) and reactive nitrogen species are formed (Siesjo *et al.* 1989). ROS include radical species – including superoxide ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and non-radical toxic species such as singlet oxygen ($O^{\cdot}{}_2$) and hydrogen peroxide (H_2O_2) (Buonocore and Groenendaal 2007), whose subsequent reactions lead to lipid peroxidation in brain cell membranes and DNA, among others, resulting in cellular damage and subsequent cell death (Bidmon *et al.* 2001).

There are several strategies of therapeutic intervention for consequences of cerebral hypoxia-ischemia in clinical and experimental approaches to reduce oxidative stress by free radicals scavenging or enhancing antioxidant power.

The primary source of highly reactive oxygen free radicals in reperfused/reoxygenated tissues appears to be enzyme xantin-oxidase (XO), formed during

hypoxia/ischemia by proteolytic attack on xantine dehydrogenase. XO converts hypoxanthine to xanthine and xanthine to uric acid. Allopurinol [4-hydroxy-pyrazole(3,4-d) pyrimidine] is a xantine-oxidase inhibitor; in high concentrations allopurinol also scavenges hydroxyl radicals and prevents free radical formation by chelating their catalyst non-protein bound iron (Mink *et al.* 1991, Kellen and Robertson 2010).

The aim of present study was to test the impact of allopurinol pretreatment on hypoxia induced changes of the brain cortex excitability. Experimental pattern of repeated electrical stimulation and analysis of afterdischarges (ADs) duration is widely accepted model for testing pro/anti-convulsive properties of different substances and is used in our laboratory for many years (Maresova *et al.* 2001, Mares and Kubova 2008). Four age groups were tested in our experiment (12, 18, 25 and 35-day-old) because of their relevance to hypoxia/ischemia event and secondly to enlighten the developmental differences (seizure-prone behaviour is age dependent) related to possible benefit of allopurinol on cortical seizure susceptibility.

Methods

Animals

All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and in agreement with the guidelines of the Animal Protection Law of the Czech Republic. Rat pups entered the experiment at postnatal day 0 (PD 0, day of birth counted as zero). There were at least 10 animals in each experimental group. Young male rats were kept with their mothers for 8 hours a day (except day 6, 7, 13 and 14) in hypobaric chamber till the PD17 (except the youngest experimental group – this group was exposed to hypoxia only till PD11). Each day, immediately before placing to hypobaric chamber, pups were pretreated subcutaneously with allopurinol (135 mg/kg; 10 mg allopurinol dissolved in 1 ml of saline) or sham-treated with equal volume of saline.

The injection was followed by exposure to 41 kPa hypobaric hypoxia (simulated altitude 7000 m), which was reached in 2 minutes (30 kPa/min) and lasted 8 hours. In-between hypoxia sessions animals were housed at a constant temperature (23±1 °C) and relative humidity (60 %) with a fixed 12 h light/dark cycle (with lights on at 07:00), under ambient pressure conditions (app. 101 kPa) and fed (or their mothers) with food and water *ad libitum*.

Electrophysiology

Electrophysiological experiments took place on PD12 (24 hours after last hypoxia procedure), 18 (24 hours after last hypoxia procedure), 25 (8 days after last hypoxia procedure) and 35 (18 days after last hypoxia procedure). On that testing day animals were transported into the experimental room, weighed, marked and randomly assigned into particular experimental groups. All tests were performed between 9 AM and 5 PM. For monitoring electrocorticogram (ECoG) and electrical stimulations six silver electrodes were implanted epidurally through the cranium under deep ether anaesthesia: two stimulation electrodes (right sensorimotor cortex), three registration electrodes (left sensorimotor cortex, left and right visual cortex) and reference electrode (placed into nasal bone). Recording and other experimental manipulations were carried out after the recovery of righting and suckling reflexes (i.e. approximately 15 min after the surgery), then the cortical afterdischarges (ADs) were elicited by stimulation of the right sensorimotor cortex. We used constant current stimulation (biphasic pulses – pulse duration of 1 ms; duration of stimulation 15 s; frequency 8 Hz; intensity 3-5 mA, which is sufficient for ADs eliciting). The basic stimulation intensity level was set at 3 mA. In case of no response, another stimulation of 4 mA was used 5 min after the first stimulation. The process was similarly repeated with 5 mA stimulation. Finally, if no epileptic graphoelements appeared after the 5 mA stimulation, the animal was excluded from the experiment. If a distinct response (epileptic graphoelements) was recorded, the stimulation was repeated five times at one-minute intervals (timed from the end of each seizure to the beginning of the next stimulation). The duration of evoked ADs and the shape of evoked graphoelements were recorded. Electrocorticograms were recorded 5 minutes before the very first stimulation and during whole stimulation process. The behaviour of rats was video-recorded.

Statistics

Differences in ADs duration between the experimental groups were compared with one-way ANOVA. For data not normally distributed, Kruskal-Wallis one-way ANOVA on Ranks and a Dunn's post hoc analysis was used.

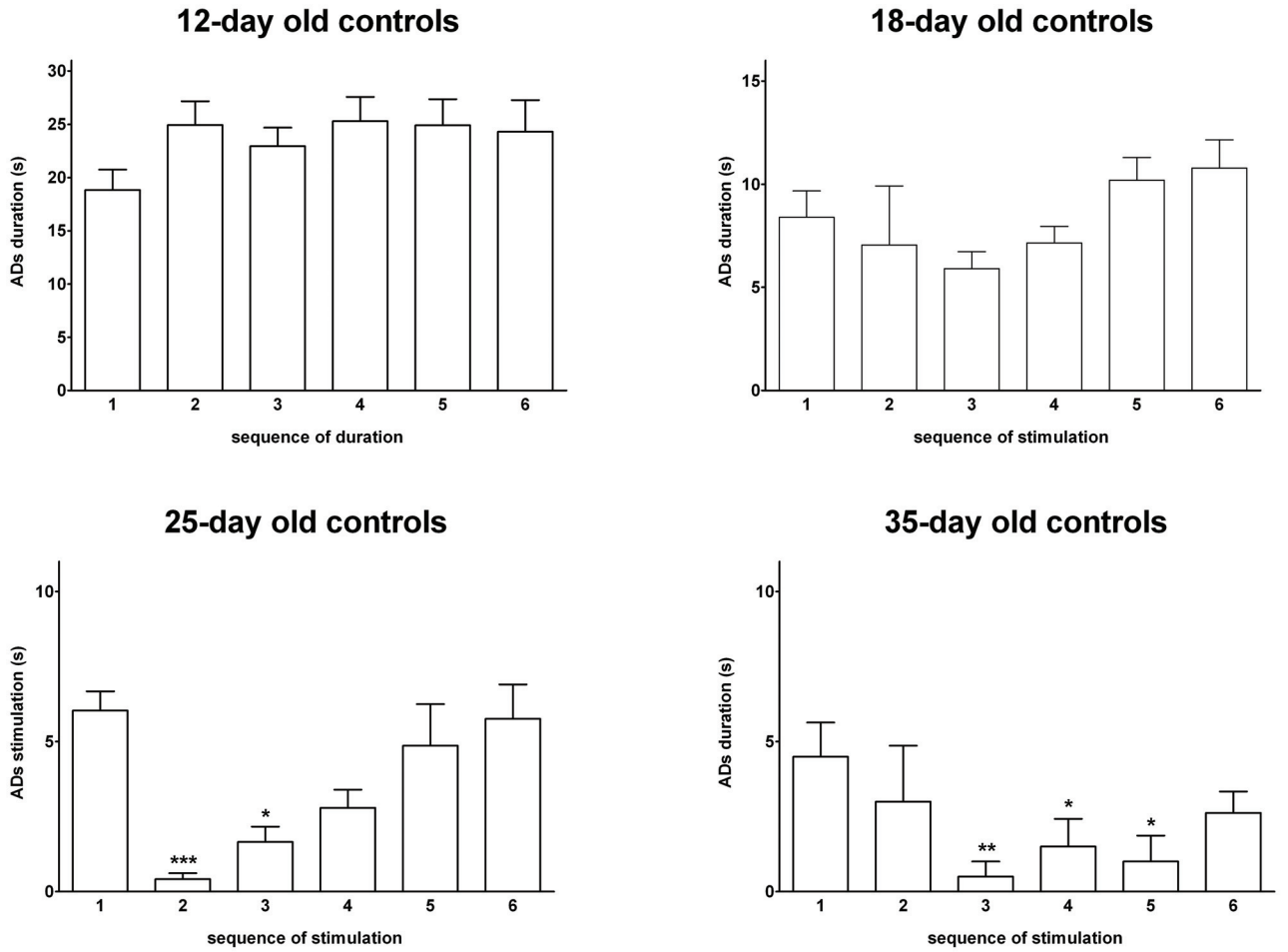

Fig. 1. Duration of ADs in 12, 18, 25 and 35-day-old rats. White columns – sham-treated animals (controls). 1-6 sequence of stimulation. Y axis represents the duration of ADs (seconds), * indicates results significant at $p<0.05$, ** indicates results significant at $p<0.01$, *** indicates results significant at $p<0.001$, mutual comparison of first ADs duration that served as baseline with the following ones.

Results

Stimulation of sensorimotor cortical area brought about forelimbs movements in the rhythm of stimulation. The ADs in 12-day-old animals were represented by rhythmic sharp waves only, while ADs in older group generated spike-and wave rhythm and this ECoG pattern (shape of discharges) did not differ between particular experimental groups. Clonic seizures that accompanied elicited ADs were synchronous with sharp ECoG graphoelements.

Control animals

Results obtained from control groups (sham treated) confirmed previously described fact that the length of ADs progressively declines with age. First ADs length in 12-day-old animals is longer (18.74±12.02) compared to 18-day-old animals (8.47±5.82, $p<0.05$), 25-day-old animals (6.03±3.46, $p<0.05$) and 35-day-old animals (4.5±3.21, $p<0.05$). In 12- and 18-day-old animals the duration of subsequent ADs (when compared with first ADs length) is unchanged. 25- and 35-day-old rats exhibited the post-ictal depression phenomenon characterized by the shortening of ADs elicited by subsequent electrical stimulation (mutual comparison of first ADs duration that served as baseline with the following ones) (Fig. 1).

Effects of hypoxia on ADs length

12-, 18-, and 25-day-old animals exposed to hypoxia exhibited the prolongation of ADs in our pattern of repeated stimulation when compared to animals not exposed to hypoxia. Effect of hypoxia was most marked in youngest experimental group (Fig. 2). Analysis of ADs duration in the oldest experimental group brought about increase in ADs length after the very first stimulation, while ADs after subsequent stimulations remained unaffected by hypoxia (except the sixth one).

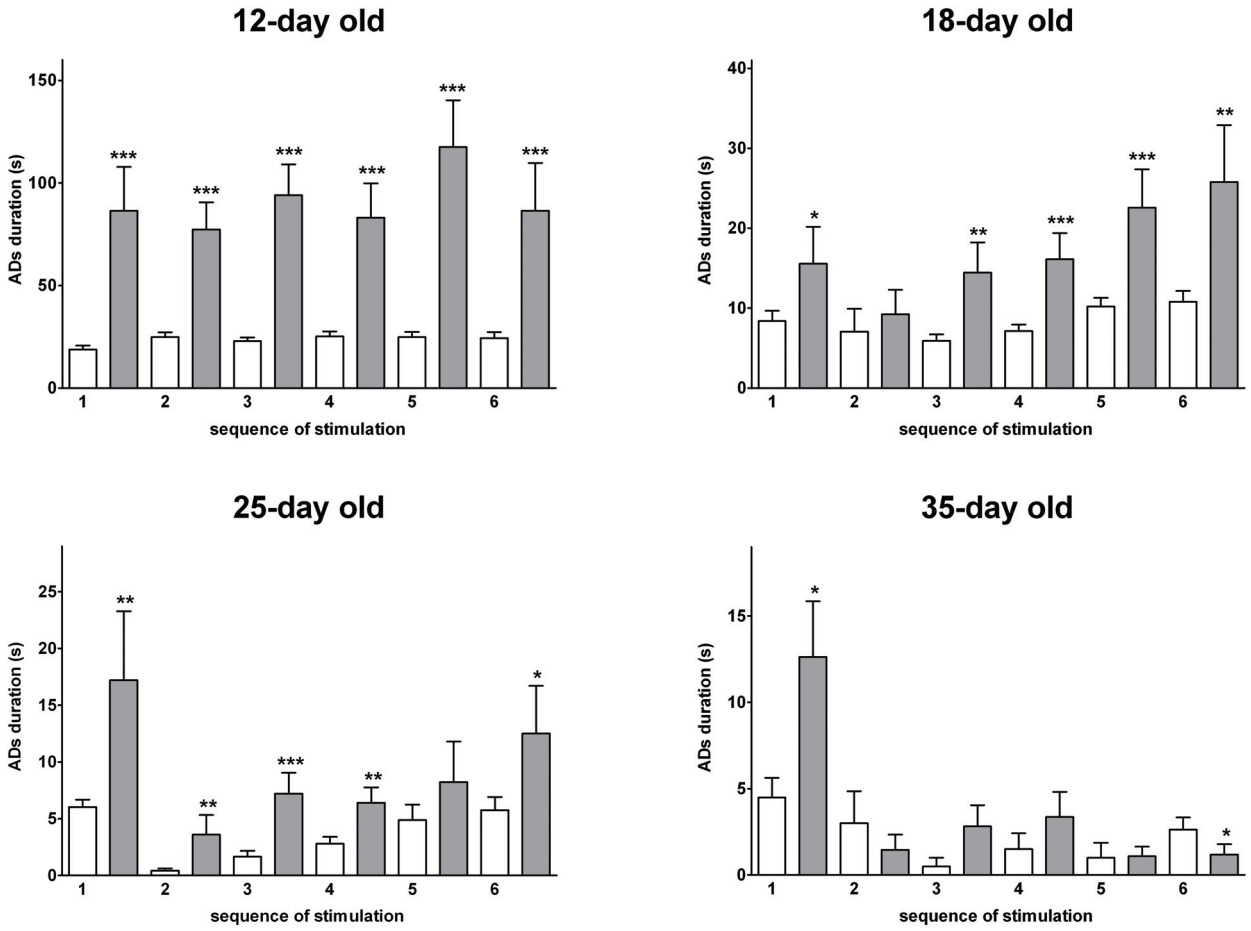

Fig. 2. Duration of ADs in 12, 18, 25 and 35-day-old rats. White columns – sham-treated animals (controls), grey columns – animals exposed to hypoxia. 1-6 sequence of stimulation. Y axis represents the duration of ADs (seconds), * indicates results significant at $p<0.05$, ** indicates results significant at $p<0.01$, *** indicates results significant at $p<0.001$, mutual comparison between controls and hypoxia exposed animals.

Effect of allopurinol pretreatment on hypoxia-induce changes of cortex excitability

Pretreatment with allopurinol significantly influenced the ADs duration in 12-day-old rats: the 1st, 3rd, 4th and 5th ADs duration were significantly shorter in comparison with those obtained from animals exposed to hypoxia. No effect was observed in 18-, 25- and 35-day old animals (Fig. 3).

Discussion

Episode of prenatal/perinatal hypoxia followed by period of reoxygenation plays important role in development of cerebral palsy, with possible serious consequence of the epileptic seizures. The aim of our study was to test hypothesis, if allopurinol pre-treatment can influence changes of brain function after repeated exposition to hypobaric hypoxia in rats during early ontogenesis. To test the susceptibility of central nervous system and possible effect of allopurinol method of evoked cortical afterdischarges was used, because it effectively tests and senses the susceptibility and excitability of brain tissue and can register changes of the brain homeostasis (Lipton 1999, Doble 1999, Golan and Huleihel 2006). This seizure-prone behavior is maintained by balance/ disbalance of excitatory and inhibitory neuronal mechanisms. It is well described that the inhibitory mechanisms are developing during the period of early ontogenesis (reflecting the development of neurotransmitter systems) and this could explain the fact, that 12- and 18-day-old animal are yet unable to prevent prolongation of ADs after repeated stimulation (Kalincik and Maresova 2005). The inhibitory systems are probably well functioning in older animals therefore suppression of ADs prolongation could be recorded in groups of older animals after the repeated stimulation. Electrical stimulation following the very first afterdischarge shorted the length of subsequent ADs. This phenomenon in 25- and 35-day-old rat is manifested as post-ictal depression and graphical representation of such relation has so called "U-shape" (Fig. 1).

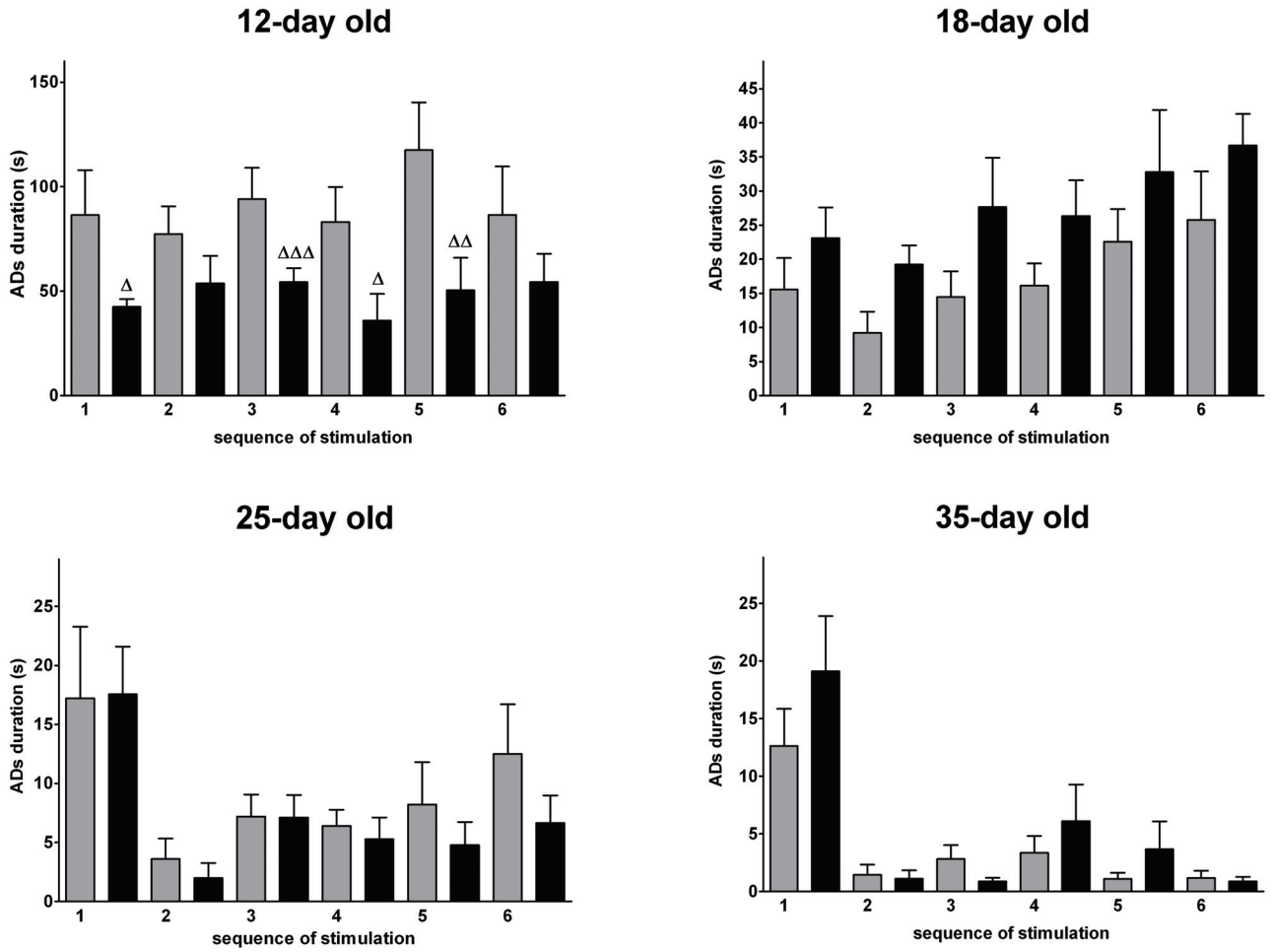

Fig. 3. Duration of ADs in 12, 18, 25 and 35-day-old rats. Grey columns – animals exposed to hypoxia, black columns – animal pretreated with allopurinol and exposed to hypoxia. 1-6 sequence of stimulation. Y axis represents the duration of ADs (seconds), $^{\Delta}$ indicates results significant at $p<0.05$, $^{\Delta\Delta}$ indicates results significant at $p<0.01$, $^{\Delta\Delta\Delta}$ indicates results significant at $p<0.001$, mutual comparison between hypoxia exposed animals and animals treated with allopurinol before each hypoxia exposition.

The repeated hypoxia in our experiment prolonged the duration of ADs in 12-, 18- and 25-day-old rats, while 35-day old animals remained unaffected; except the first and last evoked ADs (Fig. 2). This result reflects the sensitivity of immature brain tissue to hypoxia and its ability to terminate seizure efficiently (Maresova *et al.* 2001). Hypoxic episode has a severe impact on brain maturation and triggers a cascade of biochemical and molecular events, such ATP failure, membrane depolarization, alteration the ionic equilibrium, brain edema, increased neurotransmitter release, increase of intracellular calcium, production of free oxygen radicals and lipid peroxidation, that result in neuronal injury, neurodegeneration and cell death (Vannucci and Vannucci 2005, Groenendaal *et al.* 1999). It is well known, that excessive generation of free radicals is involved in excitability and seizure-related brain disturbance (Mori *et al.* 1990, Waldbaum *et al.* 2010). Because the immature brain is poor in antioxidant defence systems, many anti-oxidant drug and free radicals scavengers have been used and widely tested for their possibility to protect the neuronal tissue from excitotoxic damage. In our experimental study effect of repeated allopurinol pretreatment on hypoxia induced changes of brain cortex excitability was tested. Allopurinol is structural analogue of hypoxantine and an inhibitor of the enzyme xantine oxidase that catalyses the transformation of hypoxantine to xantine and uric acid. Allopurinol reduces purine degradation and uric acid formation and then is commonly used worldwide for the treatment of gout and hyperuricemia in human (Itoh *et al.* 1986, Saugstadt 1996). Oxypurinol is active metabolite of allopurinol that crosses the blood-brain barrier more easily than allopurinol itself (Day *et al.* 2007). Allopurinol in higher concentration also directly scavenges the hydroxyl free radicals (Das *et al.* 1987), chelates metal ions (Ko and Godin 1990) and enhances electron transport (Peterson *et al.* 1986). Allopurinol

administration has been described in many experimental and human studies, including treatment of epileptic seizures, psychiatric disorders, hypoxic-ischemic injury subsequently with period of reoxygenation/reperfusion (Tada *et al.* 1991, Wada *et al.* 1992, Murashima *et al.* 1998, Akhondzadeh *et al.* 2005, Palmer *et al.* 1990, Akdemir *et al.* 2001). Our results have shown, that only the youngest experimental group (12-day-old rats) profited from the pre-treatment of allopurinol which had significantly reduced the length of ADs in comparison to group of animal exposed to hypoxia condition without allopurinol administration. Interestingly, no effect of allopurinol on length of epileptic seizures elicited by repeated electrical stimulation was demonstrated in 18- and 25-day-old rats (Fig. 3); however both of these groups exposed to hypobaric hypoxia exhibited an increase in ADs duration compared to age matched controls. It is noteworthy that the process of ictogenesis is nonlinear with many "developmental windows" of relatively higher and lower resistance to epileptogenic stimuli (Schwartzkroin 1984) and probably at this developmental stage antioxidant used in our experiment was not able to enhance the seizure arrest and the level of cortical excitability influenced by hypoxia. In 35-day old experimental group exposed to hypoxia only the first ADs was longer when compared with controls. Explanation of such differences includes not only developmental aspects, but also the length of period in-between the hypoxia exposition and electrophysiological experiments. Supplementation with free radicals scavenger allopurinol is obviously insufficient in this developmental brain stage too. We can speculate that seizure arresting mechanisms were impaired by other factors (that remained unaffected by our treatment) rather than by excessive reactive oxygen species generation. On other hand we assume, that anti-convulsive impact of allopurinol in hypoxic animals group depends on the length of period in-between the last pre-treatment by the scavenger (the same day of last hypoxia exposition) and ADs elicitation during electrophysiological recording. This "latent" period was 8 and 18 days in 25- and 35-day-old animals, while only 24-hours in two youngest groups. Future experiments (hypoxia exposition prolonged till the 24^{th}, resp. 34^{th} day) could clarify such age-related differences.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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