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Effects of oxygen insufflation during pilocarpine-induced status epilepticus on mortality, tissue damage and seizures

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Summary
Purpose: This prospective, randomized study was performed to investigate the effects of oxygen (O_2) treatment during sustained epileptic activity on mortality, subsequent seizure frequency, and neuronal damage.
Methods: Status epilepticus (SE) was induced by intraperitoneal injection of 340 mg/kg pilocarpine, and terminated by diazepam after 40 min. During SE, rats were randomized to O_2 treatment (insufflation rate of 1.5 l/min O_2) during SE or normal air conditions. Outcome measures were SE-related mortality, seizure occurrence, mossy fiber sprouting, neuronal cell loss and expression of 27-kDa heat-shock protein (Hsp27).
Results: O_2-treated and O_2-untreated animals did not differ with respect to SE latency, diazepam dose required to stop SE. While 7/38 rats died during SE in the O_2-untreated group, very little mortality (1/38) occurred in the O_2-treated group ($P < 0.05$). However, within 1 h after SE termination, seven O_2-treated rats died which was not observed in the O_2-untreated group indicating no significant difference in overall mortality. There was a tendency towards lower seizure rate in the O_2-treated group at one month after pilocarpine-induced SE. Three months after SE, however, seizure rates were no longer different between both groups. Moreover, mossy fiber sprouting, neuronal cell loss and Hsp27 expression did not differ between O_2-treated and O_2-untreated groups.
Conclusion: Our findings indicate that O_2 treatment might delay the relative risk of epileptic seizures following an initial brain injury, but it may also lead to a rather unfavorably increased heterogeneity of epileptogenesis in experimental studies.

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Introduction

Understanding the molecular pathogenesis of temporal lobe epilepsy largely relies on the use of rodent models such as the chronic epilepsy following a sustained pilocarpine-induced status epilepticus (SE) (Turski et al., 1983). However, research in this field might be fairly troubled by SE-related mortality. Depending on the species and the pilocarpine administration protocol, mortality rates may be up to 40% (Curia et al., 2008). Hence, reducing mortality during SE is pivotal in both clinical practice and experimental epileptology. Anecdotally, oxygen (O_2) breathing via tracheotomy was once mentioned to stop status epilepticus (Niedermeyer, 1959), and as a matter of fact, current guidelines commonly include the recommendation to give supplemental O_2 insufflation in the clinical management of SE (Engrand et al., 2009). Recently, magnetic resonance (MR) imaging and MR spectroscopy performed in rats while experiencing pilocarpine-induced SE showed increased lactate and reduced choline signals in the hippocampus during SE (Van Eijsden et al., 2004). These findings were interpreted as indicative for hypoxia occurring during SE, and thereby provided experimental evidence for the potential benefit of supplemental O_2 therapy in SE. On the other hand, hypoxia has also been reported to decrease epileptic seizure activity (Kloiber et al., 1993). Therefore, the aim of this prospective, randomized study was to investigate the effect of O_2 insufflation during pilocarpine-induced SE. Outcome measures included SE survival, occurrence of spontaneous recurrent seizures, mossy fiber sprouting, neuronal cell loss and expression of 27-kDa heat-shock protein (Hsp27). While mortality and neuronal damage did not differ between O_2-treated and O_2-untreated animals, there was a tendency to reduced seizure frequency in the O_2-treated group.

Materials and methods

Pilocarpine-induced status epilepticus and oxygen treatment

The muscarinic agonist pilocarpine was used to induce a status epilepticus (SE) in male Wistar rats (30—32 days, 122 ± 31 g, mean $\pm$ SD, $n = 136$; Charles River, Sulzfeld, Germany). All procedures were performed according to national and international guidelines on the ethical use of animals (European Council Directive 86/609/EEC). In order to reduce peripheral cholinergic effects, rats were first given methyl-scopolamine nitrate (Sigma—Aldrich, 1 mg/kg, i.p.) 30 min prior to pilocarpine treatment. Then, one single administration of pilocarpine hydrochloride (Sigma—Aldrich, 340 mg/kg, i.p.) or saline (referred to as control animals) was applied, and the animals were carefully monitored to observe spontaneous seizures with progression into SE.

The onset of SE was determined when an animal had a stage 4 or 5 seizure (Racine, 1972) that was followed by continuous epileptic motor activity without showing any reaction to sensory stimuli such as gently touching against the whiskers. Immediately, the animal was randomized to one of two identical cages (55 cm $\times$ 35 cm $\times$ 19 cm), one of which was equipped with oxygen (O_2) insufflation (1.5 l/min O_2). Due to the randomization process, both animal groups had a similar mean body weight (with O_2: 125 ± 5 g, without O_2: 120 ± 5 g). The time between pilocarpine injection and SE onset was noted and no attempt was made to ameliorate the motor seizures during SE. After 40 min of SE duration, all rats received a 500 μl bolus injection of diazepam solution (Ratiopharm, Ulm, Germany, 5 mg/ml, i.p.) and were discontinued from O_2 insufflation. Occasionally, diazepam had to be re-injected in order to stop seizure activity. Importantly, the experimenter who decided to re-inject diazepam and diagnosed successful SE termination was blinded with respect to O_2 treatment or not. The total injection volume of diazepam was noted (on average 825 ± 340 μl, mean $\pm$ SD, $n = 66$), and the rats were fed with 5% glucose solution for 1 day and kept in separate cages.

Video-monitoring of spontaneous epileptic seizures

All animals were housed in identical cages in a fixed 12-h day-and-night environment (lights on from 06:00 h to 18:00 h) with food and water *ad libitum*. In order to detect the occurrence of spontaneous epileptic seizures after pilocarpine-induced SE, 24 randomly chosen rats ($n = 10$ with O_2, $n = 14$ without O_2) were transferred to a video-monitoring room at day 23 after SE, and the video-monitoring started one week later on day 30 after SE. Another subset of animals was video-monitored on day 90 after SE. Focal motor (stages 2—3) and secondarily generalized seizures (stages 4—5, Racine, 1972) were separately counted for six consecutive days (i.e. from day 31 to day 36 after SE; 12 h per day).

Mossy fiber sprouting and neuronal cell loss

All animals were decapitated in deep anesthesia with diethyl ether (Merck Biosciences) at 80—90 days after SE, and the brains were quickly removed and separated into two hemispheres. From one hemisphere, the total hippocampus was carefully dissected under a stereomicroscope, directly frozen in liquid nitrogen, and then stored at $-80\,^{\circ}C$ for Western blot analysis (see below). The other hemisphere was incubated in Na_2S-containing 0.15 M phosphate buffer at $4\,^{\circ}C$ for at least 24 h in order to stain the Zn^{2+}-positive mossy fibers in the epileptic hippocampus using standard protocols (Timm, 1958). This Timm staining was performed on horizontal cryostat sections (20 μm) using $AgNO_3$ in a citrate-buffered developer solution by an experimenter blinded with respect to O_2 treatment. Nuclei were counterstained with toluidine blue (1 g toluidine blue + 4 g $Na_2B_4O_7 \cdot 10H_2O$ in 400 ml water). For this staining, we used those animals that had been video-monitored before ($n = 16$ with O_2, $n = 22$ without O_2). The degree of mossy fiber sprouting was assessed by two measures: (i) a simple classification scheme performed by two investigators blinded with respect to O_2 treatment (0 = no staining, 1 = patches of staining in the inner molecular layer, 2 = confluent staining, 3 = massive staining). (ii) A morphometric analysis measuring the brownish-colored area within the inner molecular layer, which was normalized to the granule cell area in the same slice to produce a mossy fiber sprouting index using the ImageJ software. This procedure was performed 3 times for the same animal to yield an average mossy fiber sprouting index for each animal. Neuronal cell loss was also assessed in Timm-stained slices