Bidirectional influences of acetazolamide on central nervous system oxygen toxicity of rats

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ABSTRACT
Central nervous system oxygen toxicity, which occurs during diving and hyperbaric oxygen treatment, can lead to very dangerous situations, and it is of great importance to explore its mechanisms. We have speculated that cerebral blood flow plays a pivotal role in its occurrence. Except for acting as an anticonvulsant in clinical applications, acetazolamide is also a vasodilator used in both clinical and laboratory settings.

In this study, when acetazolamide from 5 to 500 ug/kg body weight was administered by intracerebroventricular injection, the latency of central nervous system oxygen toxicity detected by electroencephalogram recording in rats subjected to hyperbaric oxygen at 6 atmospheres absolute was prolonged significantly.

On the contrary, when the dose of intracerebroventricular injection achieved 5,000 ug/kg body weight, acetazolamide shortened the latency significantly. Intraperitoneal injection of acetazolamide more than 7.5 mg/kg body weight also shortened the latency significantly. Results also showed both intracerebroventricular injection of acetazolamide at a dose of 5,000 ug/kg body weight and intraperitoneal injection at dose of 7.5 mg/ kg body weight inhibited the activity of carbonic anhydrase and increased the cerebral blood flow significantly, which helped aggravate oxidation damage and resulted in increased MDA and impaired glutathione peroxidase in brain tissue.

But intracerebroventricular injection of acetazolamide at 5 ug/kg body weight had no effect on MDA and glutathione peroxidase, though it inhibited the activity of carbonic anhydrase.

These observations indicated acetazolamide covers bidirectional influences on central nervous system oxygen toxicity. Within local brain tissue, especially neurons, it could exert its anticonvulsive effect on the central nervous system at low doses. On the other hand, under high doses, it would display its convulsive-hastening effect through increasing cerebral blood flow to aggravate the oxidation state of brain tissues and exacerbate central nervous system oxygen toxicity when subjected to hyperbaric oxygen. Blood flow of brain plays a pivotal role in central nervous system oxygen toxicity.

INTRODUCTION
Oxygen toxicity of the central nervous system occurs in professional and military diving with closed-circuit apparatus and in diving with oxygen-enriched gas mixtures [1-3]. There is also a risk of central nervous system oxygen toxicity (CNS-OT) during hyperbaric oxygen (HBO2) medical treatment [4-7]. HBO2 therapy may be used to treat air embolism following heart surgery, gas gangrene, carbon monoxide poisoning and non-healing wounds in diabetic patients. CNS-OT is characterized by convulsions and sudden loss of consciousness, which may manifest as signs with mild neurological symptoms to severe tonic-clonic convulsions.
It is, therefore, of great importance to understand the mechanism of CNS-OT. Though many researchers have devoted efforts to explore the mechanism of CNS-OT, its mechanism has remained unclear. Some scientists found CNS-OT or HBO2-induced convulsions were usually preceded by a fluctuation in regional cerebral blood flow (CBF) [8, 9]. It also has been observed that many drugs which contract cerebral arterioles prolong the latency of CNS-OT, while those that dilate cerebral arterioles shorten the latency [8, 9].

Acetazolamide, also called diamox, plays a role as a carbonic anhydrase inhibitor and is used as an anti-epileptic agent [10]. It is well known that CNS-OT resembles epilepsy and that many anticonvulsant drugs attenuate CNS-OT [11, 12]. But it was reported that acetazolamide given by intraperitoneal (i.p.) injection shortened the latency of CNS-OT [13]. We speculated this paradox could perhaps imply an underlying mechanism for CNS-OT.

In order to test our hypothesis that cerebral blood flow regulation plays a pivotal role in CNS-OT, we have done a series of tests to explore it.

**EXPERIMENTAL PROCEDURES / METHODS**

**Animals and surgical procedures**

Male Sprague-Dawley rats (221 to 263 g, from B&K Universal Ltd, U.K.) were used. All procedures were carried out in accordance with standard guidelines for the care of animals and were approved by the Ethics Committee for Animal Experiments of the Second Military Medical University. Four to five days prior to the experiments, the rats were anesthetized with pentobarbital sodium (Nembutal, 45 mg/kg, i.p.) then fixed in a JiangWan type-1 stereotaxic apparatus. Three holes (about 0.1 mm in diameter) were drilled in the skull, and three copper electrodes were implanted. The EEG leads, copper wire with silastic insulation, were exposed by stripping approximately 0.5 mm of the insulation from the tips ends and placed in the skull holes drilled 2 mm anterior to the bregma and 3 mm right to the midline for the recording electrode (occipital position) and 4 mm posterior to the bregma and 2 mm left to the midline for the reference electrode. The grounding electrode was located near the nasal bone.

The electrodes were sealed in the holes with a small amount of acrylic cement, and the incision was closed with silk. A general penicillin antibiotic was administered (30,000 units; intramuscular) immediately after surgery, and the rats were allowed four days to recover.

For intracerebroventricular (ICV) injection, rats were anesthetized (pentobarbital sodium, i.p.) and positioned in a stereotaxic apparatus. The skin and connective tissues were removed from the skull, holes were drilled, and a diameter of 1 mm of cannula was placed into the unilateral ventricle, using stereotaxic coordinates 1 mm posterior to bregma, 1.4 mm lateral from the midline, and 4 mm below the surface of the skull. The cannula was secured with dental cement (Hy-bond Glasionomer CX, Shofu Inc., Kyoto, Japan) and anchored to the skull with two stainless steel screws. A stainless steel guide cannula (Plastics One, Roanoke, Va.) was implanted in the right lateral ventricle for ICV injection before experiments. The rats were allowed seven days of recovery.

**Latency determination**

In order to measure the latency of CNS-OT for rats treated with intraperitoneal injections of acetazolamide, 60 rats were randomly divided into six groups with 10 rats in each group. In order to measure the latency for rats treated with ICV injection of acetazolamide, 48 rats were divided randomly into six groups, with eight rats in each group.

After being placed in a 4-liter pressure chamber, the EEGs of the animals were recorded with an ADI POWERLAB/8SP and a dual-channel biology amplifier (ADInstrument company, Australia). The chamber was flushed with 100% oxygen (for about five minutes) before the pressure rose to 6 ATA (absolute atmosphere, in five minutes). A flow of 0.1 L/s of oxygen was maintained through the chamber to prevent accumulation of CO2.

The latency was determined by EEG recording. When a series of continuous spikes or sharp waves presented, we recorded the time duration as the latency. We also calculated the total time of continual convulsive EEG waves in 10 minutes after the outbreak to represent the strength and frequency of convulsive seizures. Thirty-two rats were divided into four groups.

**Activity of carbonic anhydrase**

Forty-five rats were divided into nine groups at random. After i.p. or ICV injection of acetazolamide and subjected to compression, rats were decapitated, and the cortices were homogenized. The homogenate was centrifugated at 12,000 rpm (Eppendorf, Germany) for 15 minutes. The supernatant was obtained and the carbonic anhydrase activity was detected. It was calculated through testing the time for CO2 to
decrease two (from 10.0 to 8.0) points of pH value and using the equation of:

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\text{Carbonic anhydrase activity (EU/mg protein)} = \log \left( \frac{t_b}{t_e} \right) / \text{protein} \times 10^2.
\]

The \( t_b \) means the time for boiled carbonic anhydrase and \( t_e \) represents the untouched enzyme. The system was kept in an ice-water complex, and the Lowry method was applied to test the protein.

**Cerebral blood flow measurement**

Twelve rats were divided into three groups randomly. The cerebral blood flow (CBF) of animals was recorded with a laser-Doppler flow probe and the component module of an ADI biology amplifier. After a hole was drilled in the skull, the pia mater was exposed to the probe of the laser-Doppler flowmeter, and the flowmeter was fixed on the head. Animals were put into the hyperbaric oxygen chamber, and blood flow of the cerebral pia mater was recorded with the software of Chart 5.

The difference in blood flow of the outbreak state subtracted from that of the beginning of compression was recorded for fluctuation value.

**MDA and GSH-Px measurements**

Seventy-two rats were randomly divided into nine groups. After vehicle (normal saline, NS) or acetazolamide injection, animals were subjected to a pressure of 6 ATA of pure oxygen in respect to its time course group. In view of the latency measured by EEG recording, six and 16 minutes were used as the exposure time.

After exposure the rats were decapitated, and the cortex, hippocampus and striatum of brains were dissected and homogenized. The content of maleic dialdehyde (MDA) and the activity of glutathione peroxidase (GSH-Px) were determined by the lipid peroxidation MDA assay kit and glutathione peroxidase assay kit (Nanjing Jiancheng Bioengineering Institute, China). All determinations were carried out with colorimetry.

**Statistics**

Data were expressed as the mean ± SD. All data were analyzed by ANOVA and a multiple comparison test (SNK-q). A value of \( p<0.05 \) was accepted as significant.

**RESULTS**

Acetazolamide affects the latency and strength of CNS-OT diversely under different administration pathways

We detected the latency of CNS-OT with electroencephalogram (EEG) recordings. Two sections of the EEG of an HBO_2-compressed rat were shown in Figure 1a (see Page 274). The onset of CNS-OT was characterized by a series of electricity discharges which resembled an epileptic convulsion on the EEG. All of the experimental animals displayed this.

When the dosage of the i.p. injection was more than 7.5 mg/kg body weight, the latency was shortened in a dose-dependent manner (see Figure 1b, \( *p<0.05, **p<0.01 \), Page 274).

On the contrary, when acetazolamide was administered intracerebroventricularly with a dosage of more than 5 μg/kg body weight, the latency was prolonged (see Figure 1c, \( *p<0.05 \)). But when the dosage achieved 5,000 μg/kg body weight, the latency was shortened greatly, to \( **p<0.01 \).

We calculated the total time of the convulsive EEG wave 10 minutes after outbreak (see Figure 2, Page 275). Data showed that i.p. injections of acetazolamide at 7.5 mg/kg body weight and ICV injections of 5,000 μg/kg body weight increased the convulsive time (\( **p<0.01 \)), yet ICV (5 μg) injections decrease the convulsive time (\( *p<0.05 \)).

Acetazolamide inhibits the activity of carbonic anhydrase

The i.p. injections of acetazolamide at 7.5 mg/kg body weight and ICV injections of 5,000 μg/kg body weight inhibited activity of carbonic anhydrase in brain cortices significantly (see Table 1, \( *p<0.05, **p<0.01 \), Page 275). Acetazolamide did not decrease the activity of carbonic anhydrase in six minutes when given with ICV injections at 5 μg/kg body weight (\( p=0.08 \)), yet in 16 minutes it decreased the activity of carbonic anhydrase significantly (\( *p<0.05 \)).

High dose of acetazolamide increases cerebral blood flow significantly

Acetazolamide given by i.p. injection at 7.5 mg/kg body weight increased cerebral blood flow 111.3±10.9 blood perfusion unit (BPU); ICV injections of 5,000 μg/kg body weight increased CBF about 96.32±11.79 BPU (see Figure 3, \( **p<0.01 \), Page 276).
ICV injections of acetazolamide at 5 ug/kg body weight did not produce significant differences when compared with controls.

**DISCUSSION**

Even though CNS-OT was observed more than a century ago, because of the difficulty of obtaining data live from inside a hyperbaric chamber, its mechanism remained unclear. Previous studies on HBO2-induced CNS-OT mainly involve production of active oxygen species, lipid peroxide formation and oxidation of enzyme sulphydryls [14,15,16]. In addition to these findings, a great deal of research implicated that CBF may be correlated to CNS-OT closely [4,8,9], yet the effect of HBO2 on cerebral blood flow and EEG have not been well studied and therefore the role of cerebral blood flow on convulsion outbreaks needs to be determined.

As a carbonic anhydrase inhibitor, acetazolamide was first used as an anti-convulsant. Usually used in treatment of refractory partial seizures [17], it exerted its function through inhibiting the activity of

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**FIGURE 1**

- High dose of acetazolamide increases production of maleic dialdehyde (MDA) and impairs the activities of glutathione peroxidase (GSH-Px)

High doses of acetazolamide could aggravate oxidation damage of brain tissues caused by HBO2. Except for 16 minutes in striatum, both i.p. injection of acetazolamide at 7.5 mg/kg body weight and ICV injection of acetazolamide at 5,000 ug/kg body weight increased the production of MDA significantly. It also impaired the activity of GSH-Px.

When the exposure time achieved 16 minutes, the activity of GSH-Px in the cortex and hippocampus was decreased significantly when acetazolamide was given by i.p. injection at 7.5 mg/kg body weight and ICV injection at 5,000 ug/kg body weight (see Figures 4-5, *p<0.05, Pages 277-78).

However, at six minutes, the activity of GSH-Px was improved in the diamox-treated group. ICV administration of acetazolamide at 5 ug/kg body weight did not influence MDA content and GSH-Px activity significantly.
carbonic anhydrase in brain [5,18,19]. When it was given intracerebroventricularly with the dosage of 5-500 ug/kg body weight, acetazolamide delayed the outbreak of CNS-OT induced by HBO₂, which confirmed its anticonvulsive effect. Yet when the dosage achieved 5,000 ug/kg body weight, acetazolamide shortened the latency significantly.

Intraperitoneal injection of acetazolamide also shortened the latency to CNS-OT significantly in a dose-related manner. Our data showed that both i.p. injections at 7.5 mg/kg body weight and ICV injections of acetazolamide at 5,000 ug/kg body weight could increase cerebral blood flow and aggravate oxidation damage under hyperbaric oxygen.

As it was not difficult for acetazolamide to penetrate the blood-brain barrier and take further effect on the brain, it was easy to understand i.p. injections of acetazolamide at 7.5 mg/kg body weight and ICV injections at 5000 ug/kg body weight displayed like effects on CNS-OT. Notwithstanding, as mentioned above, acetazolamide at 5-500 ug/kg body weight ameliorated CNS-OT and did not influence cerebral blood flow and oxidation damage under hyperbaric oxygen.

These results indicated that there should be bi-directional influences of acetazolamide on CNS-OT. The first one was that acetazolamide exerted its function as one kind of antiepileptic medicine in CNS-OT under low dosages, which resembled its effect in
treating epilepsy. The other one was through acting as a dilator of the brain arterioles to aggravate the oxidation by increasing the oxygen supply when given intraperitoneally or intracerebroventricularly in high doses.

The effect of dilating brain vessels and aggravating oxidation damage suggested the outbreak of CNS-OT literally correlated with the CBF closely. Acetazolamide’s effect on CBF could last for several hours [20, 21]. Our results also matched their finding. At the beginning of HBO₂ treatment, both in the ICV injection group and the control group, the CBF of rats decreased gradually, even below baseline level. But in rats treated with i.p. injections of acetazolamide at 7.5 mg/kg body weight and ICV injections at 5,000 ug/kg body weight, the CBF was improved significantly. More cerebral blood flow meant more oxygen supply.

Under HBO₂ and high oxygen partial pressure, the increased oxygen physically dissolved in the blood was the dominant proportion of the oxygen increment,
which played a major role in the clinical treatment and also led to oxygen toxicity [22, 23]. It was widely accepted that the fundamental reason for CNS-OT is oxidation damage: It was easy to understand that the increment of cerebral blood flow, which carried more oxygen with high partial pressure, would cause more severe oxidation damage. Our result confirmed that when the cerebral blood flow was augmented by acetazolamide the lipid peroxidation was secondarily exacerbated and GSH-Px activity was impaired, mainly in the cortex and hippocampus. It is well known that cortex neurons, especially motor neurons, are important for movement. And hippocampus also related to epilepsy closely [24,25]. Latency for peritoneal injections was consistent with a previous report that noted that acetazolamide shortened the latency of HBO₂-induced convulsion [13], a view not shared by another report [26]. The mechanism to explain such results remained controversial.

Our results provided another resolution: It was likely through dilating brain arterioles with high doses of acetazolamide that aggravated oxidation damage shortened the latency to CNS-OT. Our finding implied that acetazolamide’s convulsive-hastening effect – which was through increasing cerebral blood flow – outweighed its anticonvulsive effect when the dosage administered intraperitoneally exceeded 7.5 mg/kg body weight or intracerebroventricularly
achieved 5,000 ug/kg body weight. It was reasonable that when it was given intracerebroventricularly, acetazolamide would directly behave as an anticonvulsant and act mostly locally on neurons or glia. When its dosage was small, icV injection of acetazolamide could not affect cerebral flood significantly and only acted as an anticonvulsant. But when the dosage was large enough, since acetazolamide was permeable to the blood-brain barrier [27], icV injections of acetazolamide could also dilate the brain arterioles to aggravate the oxidation damage.

Intraperitoneal injection of acetazolamide would inevitably act on both vascular tissues and neuronal cells as well. Based on its powerful dilation on brain arterioles, acetazolamide exacerbated central nervous system oxygen toxicity although it was playing an anticonvulsive role at the same time. From the dose-dependent curve of acetazolamide, it appeared that low concentrations did not affect or even delay the latency of convulsion (its effect displays similarly to some anticonvulsants). To some degree, cerebral blood flow displayed a more powerful influence.

ICV injection of acetazolamide with proper dosage displayed anticonvulsive effects in our current study, but its precise mechanism for countering to CNS-OT needs to be further explored. In addition, more attention should be paid to those patients who receive HBO2 treatment and are given acetazolamide at the same time.
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