Preventive effects of ketone ester BD-AcAc₂ on central nervous system oxygen toxicity and concomitant acute lung injury

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Key words

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Abstract

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Background: Recent studies indicated that ketone ester R,S-1,3-butanediol acetoacetate diester (BD-AcAc₂) may be effective in preventing central nervous system oxygen toxicity (CNS-OT) and concomitant acute lung injury, a serious medical problem to be faced when breathing hyperbaric oxygen (HBO). This study aimed to further investigate the protective effects of BD-AcAc₂ against CNS-OT and concomitant acute lung injury (ALI) in mice.

Methods: Mice were treated with BD-AcAc₂ in peanut oil vehicle (2.5, 5.0 or 10.0 g·kg⁻² body weight) by gavage 20 minutes before 600 kPa HBO exposure. Control mice received the vehicle only. Seizure latency was recorded. Malondialdehyde content in brain and lung tissues, total protein level in bronchoalveolar lavage fluid (BLF) and lung water content were measured 60 minutes after the hyperbaric exposure. Histopathology of lung tissue was undertaken.

Results: Compared with the vehicle alone, BD-AcAc₂ prolonged seizure latency in a dose-dependent manner ($P < 0.01$). The HBO-induced increase in brain malondialdehyde, BLF protein and lung water were significantly reduced by BD-AcAc₂ ($P < 0.01$).

Conclusion: Oral administration of the ketone ester BD-AcAc₂ significantly protected against CNS-OT and concomitant ALI. Alleviation of oxidative stress may be one underlying mechanism providing this effect.

Introduction

Hyperbaric oxygen (HBO) treatment is widely used in the treatment of a variety of medical conditions and diving related illnesses.¹ It has been demonstrated that HBO alleviates tissue hypoxia and stimulates endogenous protection mechanisms, including expression of cytoprotective proteins thus enhancing cellular tolerance against harmful stimuli.^{2,3} To a certain extent, the therapeutic effect of HBO is positively correlated to dose (a function of pressure and duration of exposure). However, HBO may also lead to oxygen toxicity (OT), whose likelihood is also positively correlated to inspired oxygen pressure and duration. OT is one of the most concerning complications of HBOT and the dose of HBO is therefore limited in clinical practice.

OT results from the harmful effects of breathing oxygen (O₂) at elevated partial pressures.⁴ Depending on the inspired O₂ pressure and duration, different types of OT may occur. Long-term exposure to elevated O₂ levels, even at normal atmospheric pressure, may affect the lungs, eyes and other organs. Short-term exposure to a partial pressure of O₂ greater than at normal atmospheric pressure may lead to central nervous system OT (CNS-OT), which is of great

concern in both diving operations and during HBOT. With little or no warning, CNS-OT can result in a grand mal convulsion and, if occurring during diving, in drowning and death.^{5,6} Development of strategies to prevent CNS-OT could improve the safety of HBOT and diving operations.

Previous studies have reported that fasting and a ketogenic diet (KD) can inhibit refractory seizures, an effect which might be associated with increased ketone in the blood.⁷⁻⁹ Administering the KD has been reported to completely abolish seizures in up to 13% of patients and reduce seizure frequency by > 50% in approximately 67% patients with refractory epilepsy.¹⁰ The KD is now well established as a preventative measure for seizures. Convulsions resulting from CNS-OT share many common pathophysiological mechanisms with other clinical seizures. Some reports have shown that KD can delay the onset of CNS-OT.^{11,12} However, in order to be effective, the diet needs to have been established over a long period and needs strict dietary compliance that can impede its successful use.

A recent study reported that oral administration of ketone ester BD-AcAc₂ could mimic the effects of a KD and induce ketosis.¹³ It could also inhibit seizures and delay CNS-OT

Figure 1
Structure of BD-AcAc₂

onset in rats.¹⁴ In the present study, the protective effects of BD-AcAc₂ on CNS-OT, including concomitant acute lung injury (ALI) were evaluated, as were any possible antioxidative mechanisms underlying any protective effects.

Methods

The experimental procedure was approved by the Institutional Animal Care and Use Committee of the Naval Medical University, Shanghai (number 201711270007). All efforts were made to minimize suffering to the animals.

ANIMALS AND COMPOUND BD-ACAC₂

Male mice (C57BL/6) (20 ± 2 g) were provided by the animal centre of the University and housed in a temperature (24 ± 1°C) and humidity (54 ± 2%) controlled environment with a 12/12 h light/dark cycle and free access to food and water. Compound BD-AcAc₂ (Figure 1) was synthesized by transesterification and verified by proton nuclear magnetic resonance (1H NMR) and mass-spectrometry.

GROUPS AND TREATMENT

After being fasted for 18 h, mice were randomly divided into six groups (*n* = 8): Normal group, Null group, Vehicle group and treatment groups receiving three different doses of BD-AcAc₂. BD-AcAc₂-treated mice received BD-AcAc₂ in 0.4 ml of a peanut oil vehicle by gavage in a dose of 2.5, 5.0, or 10.0 g·kg⁻¹ body weight, and the Vehicle group mice received 0.4 ml peanut oil alone. The Normal group did not receive any treatment and was used as control. The Null group was exposed to HBO but received neither the vehicle nor BD-AcAc₂. The Null, Vehicle and three Treatment groups were exposed to HBO (see below). In the Vehicle and Treatment groups this occurred 20 minutes (min) after gavage with the vehicle or vehicle + BD-AcAc₂ respectively. All mice were anaesthetized 60 min following HBO exposure with 1% pentobarbital sodium (50 mg·kg⁻¹ body weight) intraperitoneally before sampling.

HBO EXPOSURE

The mice were placed singly in an animal compression chamber (RDC150-300-6, Naval Medical University,

Shanghai, China). The chamber was flushed with pure oxygen for 5 min before being compressed to 600 kPa. The compression and decompression were both carried out at a rate of 100 kPa·min⁻¹. The previously used exposure duration of 30 min was adopted.^{15,16} However, if the convulsion latency was longer than 30 min in the treated mice, the exposure was continued until the onset of a convulsion. A continuous oxygen flow of 0.5 L·min⁻¹ was maintained during the exposure and soda lime was placed in the bottom of the chamber to prevent the accumulation of CO₂.

CNS-OT LATENCY

During the HBO exposure the behaviour of the mice was observed. The convulsion latency was the elapsed time from achievement of a chamber pressure of 600 kPa to the occurrence of tetanic contraction of whole body and persistent spasm.¹⁷

BRONCHOALVEOLAR LAVAGE PROTEIN

After midline laparotomy, the abdominal aorta was cut and the mouse was exsanguinated. After isolation of the right lung, the left lung was lavaged with 0.3 ml precooled phosphate buffered saline (PBS) three times. The lavage fluid was then collected and centrifuged at 1500 g for 10 min; the supernatant was stored at -80°C. The protein in the supernatant was measured using a bicinchoninic acid (BCA) kit (Beyotime, Haimen, China).

LUNG WATER CONTENT

The superior and inferior lobes of the right lung were collected. The water on the lung surface was dried with blotting paper and the lung was weighed after drying in a 60°C oven for 72 h. Lung water content (%) was calculated as:

$$\text{Wet weight} - \text{Dry weight} / \text{Wet weight} \times 100. \quad (1)$$

MALONDIALDEHYDE (MDA) IN BRAIN AND LUNG

The brain and middle lobe of the right lung were washed with precooled saline and stored at -80°C. Fifty milligrams of the tissue was lysed with western and cell lysis buffer (Beyotime, Haimen, China). Homogeneous lysates were centrifuged at 1600 g for 10 min and the supernatant was measured using a BCA kit. An MDA Kit (Beyotime, Haimen, China) was then used to assay MDA as an index of oxidative injury to the brain and lung.

HAEMOTOXYLIN AND EOSIN (H&E) STAINING

The post-caval lobe of the right lung was fixed with 4% paraformaldehyde followed by paraffin embedding. The tissue was then sectioned followed by H&E staining. The sections were examined by plain microscopy to look for tissue inflammation, oedema or other damage.

Figure 2

Latency of CNS-OT in BD-AcAc₂ treated mice; data are expressed as mean $\pm$ SD, $n = 8$; ** $P < 0.01$ vs. Vehicle group

Figure 3

Total protein in bronchoalveolar lavage fluid after HBO exposure; data are expressed as mean $\pm$ SD, $n = 8$; ** $P < 0.01$ vs. normal group; ## $P < 0.01$ vs. Vehicle group

Figure 4

Water content (expressed as a percentage of total weight) in lung tissue after HBO exposure; data are expressed as mean $\pm$ SD, $n = 8$; ** $P < 0.01$ vs. Normal group; ## $P < 0.01$ vs. Vehicle group

Figure 5

MDA content in cerebral cortex and lung tissues after HBO exposure; data are expressed as mean $\pm$ SD, $n = 8$; ** $P < 0.01$ vs. Normal group; ## $P < 0.01$ vs. Vehicle group

STATISTICAL ANALYSIS

The data were analyzed with SPSS21.0 software. All data were presented as mean $\pm$ standard deviation (SD), and the differences between groups were compared with one-way ANOVA. $P < 0.05$ was taken to indicate statistical significance.

Results

Compared with the vehicle group, BD-AcAc₂ significantly prolonged the seizure latency of CNS-OT in a dose dependent manner ($P < 0.01$). There was no difference between the Vehicle and the Null group (Figure 2) suggesting that the vehicle had no role in the beneficial effect of BD-AcAc₂.

Total protein levels in the bronchoalveolar lavage fluid were significantly increased by HBO exposure in the Null and Vehicle groups ($P < 0.01$) whereas pretreatment with

BD-AcAc₂ significantly reversed this effect ($P < 0.01$) (Figure 3). Similarly, HBO exposure significantly elevated the water content in lung tissues ($P < 0.01$), whereas pretreatment with BD-AcAc₂ significantly decreased this effect ($P < 0.01$) (Figure 4).

MDA content in brain tissue was significantly increased after HBO exposure ($P < 0.01$) and this was reversed by BD-AcAc₂ at all tested doses ($P < 0.01$). The HBO exposure did not affect lung MDA content (Figure 5).

Staining and microscopy revealed significant lung damage in the Vehicle group, which comprised congestion in the bronchial wall and alveolar tissue, inflammatory cell infiltration and lung tissue structure distortion. This was substantially alleviated in groups receiving BD-AcAc₂, with the only change noted being pulmonary vasodilation (Figure 6).

Figure 6

Examples of lung histology (haematoxylin and eosin staining) after HBO exposure; A – Normal group (No intervention); B – Null group (HBO only); C – Vehicle group HBO and drug vehicle (see test); D – BD-AcAc₂ 2.5 g·kg⁻¹; E – BD-AcAc₂ 5.0 g·kg⁻¹; F – BD-AcAc₂ 10.0 g·kg⁻¹; magnification x 40

Discussion

A previous study showed that fasting delayed the latency of CNS-OT; an effect which appears to be related to changes of brain energy metabolism induced by blood ketones.¹⁸ The KD mimics the metabolic state of fasting (i.e., therapeutic ketosis) and has proved to be a potential preventive measure for convulsions.¹⁹ However, as the KD requires strict compliance and long-term preparation, its usefulness for this purpose in practice is greatly restricted. Recently, some preliminary studies suggested that oral administration of the ketone ester BD-AcAc₂ could mimic the KD and delay the onset of CNS-OT in rats.^{13,14} In the present study, these findings were confirmed in a mouse model and the underlying mechanisms were further explored from the perspective of protecting against oxidative injury and the effects of BD-AcAc₂ on concomitant ALI were assessed.

The ketone ester BD-AcAc₂ and its resulting metabolic compounds might contribute to a range of mechanisms that could delay the onset of OT. Oral administration of BD-AcAc₂ would generate acetoacetate (AcAc) and β -hydroxybutyrate (β HB); then part of the AcAc would be decarboxylated to become acetone.¹⁴ All three of these resulting compounds could then easily pass through blood brain barrier via a monocarboxylic acid transporter.²⁰ The mechanisms by which BD-AcAc₂ exerts its anti-OT effect might be associated with these increased ketone bodies in

the blood. It is widely accepted that increased production of reactive oxygen species (ROS) makes a major contribution to the development of CNS-OT.⁶ Ketone body metabolism can improve the function of mitochondria and reduce ROS production, thus inhibiting ROS-related cell damage and reducing oxidative stress injury.^{21,22}

Ketone bodies might also be associated with adenosine metabolism. Therapeutic ketone bodies have been reported to significantly inhibit seizures in mice, but not in A1 adenosine receptor (A₁R) knockout mice.²³ Ketone bodies can increase adenosine content and A₁R activity in the brain, reduce adenosine kinase (ADK) activity and inhibit the release of the excitatory neurotransmitter glutamate.²⁴ It is also known that the balance of glutamate (Glu) and γ -aminobutyric acid (GABA) plays an important role in the pathophysiological process of seizures. AcAc may not only inhibit Glu release²⁵ and promote its conversion to GABA²⁶, thus reducing neuronal excitability, but it may also enhance the anti-oxidation ability of hippocampal neurons and inhibit seizure activity.²⁷ β HB has a similar structure to GABA; it may mimic the effects of GABA and reduce neuronal excitability.²⁸

The present study showed that oral administration of BD-AcAc₂ significantly delayed the onset of oxygen convulsions in a dose dependent manner. The strongest effect accrued from the highest dose (10.0 g·kg⁻¹ body

weight), in which the mean convulsion latency was substantially extended from 9.6 min to 39.8 min. Exposure to HBO significantly increased levels of MDA; a sensitive indicator of oxidative stress injury in brain tissue. BD-AcAc₂ significantly reversed this change, suggesting (though not proving) that alleviation of oxidative stress may be the underlying mechanism of delaying CNS-OT. As it is proven that CNS-OT is a self-limiting disease and does not cause brain tissue histopathological changes,^{29,30} brain tissue H&E staining was not performed in this study.

The mechanism of CNS-OT concomitant ALI is far from clear. Studies have shown that excessive oxygen exposure can increase ROS production in the lung, which may result in cell injury, increased endothelial permeability and eventually pulmonary oedema.³¹ However, the role of oxidative injury in CNS-OT concomitant ALI has not been properly studied. In our study, microscopy on stained lung sections from Null or Vehicle group mice showed that 30 min of 600 kPa HBO exposure resulted in severe lung damage manifest as oedema and congestion, accompanied by inflammatory cell infiltration and lung tissue structure distortion. In contrast, BD-AcAc₂-treated mice only displayed slight pulmonary vasodilatation. These results indicated that BD-AcAc₂ was effective in CNS-OT concomitant ALI prevention. However, as MDA content in lung tissue did not increase after HBO exposure, this may indicate that oxidative injury did not play a role in CNS-OT concomitant ALI CNS-OT. Concomitant ALI has been associated with a sharp increase in pulmonary vascular pressure.³² Toxic levels of HBO exposure can lead to a large sympathetic activation of the central nervous system, resulting in the occurrence of pulmonary hypertension. Concurrently, sympathetic activation can release a large amount of catecholamine, disturbing the autonomic nervous balance and resulting in acute lung injury.³³ Adenosine may also play an important role in preventing this pathological process. Studies have shown that adenosine can significantly reduce lung surface haemorrhage and tissue damage, reduce lung tissue leakage, reduce pulmonary oedema, and effectively alleviate acute lung injury induced by oxygen convulsions.³⁴ Ketoesters may work by modulating adenosine, as discussed above. However, whether this is the underlying protective mechanism needs further investigation.

In conclusion, this study demonstrated that oral administration of the ketone ester BD-AcAc₂ could significantly protect mice from CNS-OT and concomitant ALI. However, the underlying mechanisms remain unclear, so further investigations are needed to provide more evidence for its possible future application in diving operations and HBOT.

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