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The Arousal Effect of Hyperbaric Oxygen through Orexin/hypocretin An Upregulation on Ketamine/ Ethanol-Induced Unconsciousness in Male Rats

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Abstract

Approaches that facilitate the recovery from coma would have enormous impacts on patient outcomes and medical economics. Orexin-producing neurons release orexins (also known as hypocretins) energy-dependently to maintain arousal. Hyperbaric oxygen (HBO) could increase ATP levels by preserving mitochondrial function. We investigated, for the first time, the arousal effects of HBO and orexins mechanisms in a rat model of unconsciousness induced by ketamine or ethanol.

A total of 120 Sprague-Dawley male rats were used in this study. Unconsciousness was induced either by intraperitoneal injection of ketamine or ethanol. The HBO treatment (100% O₂ at 3 ATA) was administered immediately after unconsciousness induction for 1 hour. SB334867, orexin-1 receptor (OX1R) inhibitor or JNJ10397049, orexin-2 receptor (OX2R) inhibitor was administered 30 minutes intraperitoneally before unconsciousness induction. Loss of righting reflex test (LORR) and Garcia test were used to evaluate the unconsciousness duration and

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AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Conceptualization*, B.H., L.H., Q.H., B.L. and J.Z.; *Methodology*, B.H., Q.H., B.L. and X.L.; *Investigation*, B.H., Q.H., B.L. and X.L.; *Formal Analysis*, B.H., Q.H. and L.H.; *Resources*: J.Z.; *Writing – Original Draft*, B.H., L.H. and J.Z.; *Writing – Review & Editing*, B.H., L.H. and J.Z.; *Supervision*, Q.H. and J.Z.; *Funding Acquisition*, B.H. and J.Z.

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CONFLICTS OF INTEREST

None.

neurological deficits after recovering from unconsciousness, respectively. Enzyme-linked immunosorbent assay was used to measure brain tissue ATP and orexin A levels.

Ketamine or ethanol injection resulted in LORR immediately and neurological deficits 6 hours after unconsciousness induction. HBO treatment significantly reduced the LORR duration, improved Garcia scores and unregulated ATP and orexin A levels in the brain tissue. Administration of OX1R inhibitor or OX2 R inhibitor abolished arousal and neurological benefits of HBO.

In conclusion, HBO exert arousal-promoting effects on unconscious rats induced by ketamine and ethanol. The underlying mechanism is via, at least in part, ATP/orexin A upregulation. HBO may be a practical clinical approach to accelerate unconsciousness recovery in patients.

Graphical Abstract

Keywords

Arousal; hyperbaric oxygen; unconsciousness; ATP; orexin receptor; hypocretins; rat; MyBioSource RRID:SCR_013493; SigmaPlot RRID:SCR_003210; GraphPad Prism RRID:SCR_002798

INTRODUCTION

Coma is a prolonged state of unconsciousness that commonly occurs as a result of acute severe brain injuries or metabolic disorders (Moore and Wijdicks, 2013; Rao, 2016; Schiff and Plum, 2000; Tokuda et al., 2003). In addition to various therapeutic strategies targeting multiple pathological processes in brain injuries (An et al., 2016; Kinouchi et al., 2018; Pena and Borlongan, 2015; Wang et al., 2018; Yang et al., 2018; Yu et al., 2017; Zhang et al., 2018), approaches that specifically facilitate the recovery from coma would have enormous impacts on patient outcomes and medical economics. Previous studies demonstrated that the application of appropriate arousal procedures including hyperbaric oxygen (HBO), physical therapy and arousal drugs, improved recovery of consciousness in patients with prolonged coma (Jiang et al., 2004). Although HBO have been shown to be neuroprotective in the setting of brain injury (Cossu, 2014), its direct effect on arousal and underlying mechanism are not clear.

By binding G protein coupled receptors including orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R), orexin neuropeptides, also known as hypocretins, regulate the vigilance states (Jia et al., 2012; Ohno and Sakurai, 2008; Sakurai, 2002, 2005). Orexin-producing neurons are exclusively localized to the lateral and posterior hypothalamic area, from which these cells produce a wide projection to the whole cerebrum neuroaxis (Sakurai, 2005). Intracerebroventricular administration of orexin A to the brain has been shown to increase wakefulness in normal rats (Hagan et al., 1999) and in rats subjected to alcohol intoxication-induced unconsciousness (Jia et al., 2012). The membrane potential (Vm) of orexin neurons correlated with the intracellular level of adenosine triphosphate (ATP), suggesting their functionality dependence on the energy level (Liu et al., 2011). Hyperbaric oxygen (HBO) could increase ATP levels by preserving mitochondrial function following TBI in rats (Zhou et al., 2007). Thus, HBO may enhance the orexin neurons activities through ATP up-regulation, leading to improved arousal status subsequently.

In the present study, we evaluate the arousal effects of HBO in unconscious rats induced by ketamine or ethanol in vivo. The orexin A mechanism underlying this effect was further investigated using OX1R and OX2R antagonists.

MATERIALS AND METHODS

Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University. One hundred and eighty Sprague-Dawley male rats (280-320g, Harlan Laboratories, Indianapolis, IN) were used for this study. All male rats were pair-housed in a vivarium for a minimum of 5 days prior to experiment with a 12-hour light-dark cycle and free access to food and water. The standard holding room temperature was $74\pm 2^{\circ}\text{F}$. Standard air changed 10-15 air changes/hour.

Experimental Design

Two separate experiments were conducted (Fig. 1).

Experiment 1: Ketamine experiment—Forty rats were randomized into the following 5 groups with $n=8/\text{group}$: Ketamine+Air, Ketamine+HBO, Ketamine+HBO+vehicle, Ketamine+HBO+SB334867 and Ketamine+HBO+JNJ10397049. Unconsciousness was induced by intraperitoneal (i.p.) injection of ketamine at 100mg/kg.

Experiment 2: Ethanol experiment—Forty rats were randomized into the following 5 groups with $n=8/\text{group}$: Ethanol+Air, Ethanol+HBO, Ethanol+HBO+vehicle, Ethanol+HBO+SB334867 and Ethanol+HBO+JNJ10397049. Unconsciousness was induced by i.p. injection of ethanol at 3.5g/kg (Jorgensen and Hole, 1981). To confirm the arousal effect of HBO in a severe degree of ethanol induced unconsciousness, a separate cohort of forty rats were randomized into the same 5 groups ($n=8/\text{group}$), but unconsciousness was induced by the injection of higher dose of ethanol at 4.0g/kg (Yorulmaz et al., 2011).

Each rat was taken out from home cage and put into a separate empty cage for ketamine or ethanol injection. Based on the preliminary study, it took about 3 minutes for the rats to lose consciousness after the injection of ketamine or ethanol. The exclusion criteria included 1) rats that lost consciousness longer than 4 minutes or short than 1 minute; 2) rats were not awake and died due to anesthesia accident.

HBO Treatment

HBO treatment was administered immediately after unconsciousness induction. Upon confirmation of LORR, the rats in HBO treatment group were placed into the HBO chamber with back lying on the chamber floor. Previous HBO studies have applied HBO regimen ranging from 1.5-3.0 atmospheres absolutes (ATA) for 60-90 minutes in a variety of rodent model of neurological diseases, which demonstrated the neuroprotective effect in the absence of oxygen toxicity (Zhang et al., 2005). Thus, in the present study, rats were pressurized in a research hyperbaric chamber for small animal research (1300B; Sechrist, Anaheim, CA, USA) at 3.0 ATA of 100% oxygen at 22 L/min flow rate for 1 hour. Compression and decompression were completed at a rate of 5 psi/min. After the completion 1 hour HBO treatment, the rats were taken out and put back into their home cages.

Orexin Receptors Inhibitors

SB334867, OX1R inhibitor (10mg/kg, CAS No. 792173-99-0, Sigma-Aldrich, St. Louis, MO, USA) or JNJ10397049, OX2R inhibitor (5mg/kg, Cat No. 4317, Tocris Bioscience, UK) was administered i.p. 30 minutes before unconsciousness induction. The inhibitor doses were modified from previous studies (Dugovic et al., 2009; Martin-Fardon and Weiss, 2014). In the vehicle group, rats were injected i.p. with 10% dimethyl sulfoxide (DMSO, vehicle) 30 minutes before unconsciousness induction.

Neurological Function Assessment

The neurological function was assessed by an investigator blinded to the group information.

Loss of Righting Reflex Test—Loss of righting reflex (LORR) was defined as the failure to correct the posture while lying on its back (Jia et al., 2012). In each rat, the duration of unconsciousness was assessed by the time between LORR and the recovery of righting reflex. The ambulation was recorded manually. If the rats in the HBO treatment groups recovered from unconsciousness during HBO treatment, the duration of LORR was recorded when the rats were observed to correct their back lying posture spontaneously in the HBO chamber. After the completion of 1 hour HBO treatment, the rats were took out and put back to their home cages.

Neurological Scores—Modified Garcia test was performed 6 hours after unconsciousness induction to assess neurological deficits as previously described (Garcia et al., 1995). The scores given to each rat after completion of the evaluation was the summation of 7 individual test scores (spontaneous activity, symmetry in the movement of four limbs, forepaw outstretching, climbing, body proprioception, response to vibrissae touch, and beam walking). The neurological scoring ranged from 3 (most severe deficit) to maximum 21 (normal). It took 5 minutes to perform the Garcia test in each rat.

Enzyme-linked Immunosorbent Assay (ELISA) for ATP and Orexin A

Rats were euthanized after the completion of neurological assessments. After rats were deeply anesthetized with 5% isoflurane until there was no neurological response to painful stimuli, they were perfused by phosphate buffered saline (PBS) transcardially followed by guillotine decapitation. The whole brain was extracted and further dissected into the regions of hypothalamus and somatosensory cortex. The somatosensory cortex is the projection area of hypothalamus orexin-producing neurons (Jin et al., 2016). The somatosensory cortical layer 6b was a specific target of orexin, the arousal-promoting transmitters (Bayer et al., 2004). ATP and Orexin A levels (n=6/group) were determined using ELISA kit (Cat No. MBS723034 and MBS762821, MyBioSource, Inc, Canada, MyBioSource, RRID:SCR_013493)). Because the results of LORR and neurological between ketamine/ethanol+HBO and ketamine/ethanol+HBO+vehicle were comparable, we used four groups in each experiment for ELISA measurement, namely, Ketamine/ethanol+Air, Ketamine/ethanol+HBO, Ketamine/ethanol+HBO+SB334867 and Ketamine/ethanol+HBO+JNJ10397049. Tissues were homogenized by sonication in an ice-cold phosphate buffer (pH 7.4) and centrifugated for 15 minutes at 1500×g (or 5000 rpm). Enzyme immunoassay was carried out following manufacturer's instructions.

Statistical Analysis

All analyses were performed using Sigma Plot 11.0 (Systat Software, Inc., SigmaPlot, RRID:SCR_003210) and GraphPad Prism 6 (GraphPad software, Inc., GraphPad Prism, RRID:SCR_002798). Data were expressed as the mean $\pm$ SD. For the data that passed the normality test and equal variance test, the statistical differences among groups were further analyzed using oneway ANOVA followed by Tukey multiple comparison *post hoc* analysis. Measurements of hypothalamus ATP in ethanol induced unconsciousness, hypothalamus orexin A in both ketamine and ethanol induced unconsciousness failed the normality test, thus Kruskal-Wallis One Way Analysis of Variance on Ranks were used followed by Tukey multiple comparison *post hoc* analysis. Pearson R Bivariate correlation was used to analyze the correlation between orexin A with LORR in HBO treated unconscious rats. *P* value of less than 0.05 was considered statistically significant.

RESULTS

All rats survived from unconsciousness induction and HBO treatment. No rats were excluded from the study.

Arousal Effects of HBO

In all rats, LORR was observed about 3 minutes after the injection (i.p.) of ketamine at a dose of 100mg/kg, ethanol at a dose of 3.5g/kg or ethanol at a dose of 4.0g/kg. Compared to the air treated group, HBO significantly decreased the duration of LORR and improved Garcia scores 6 hours after recovery of righting reflex. Pretreatment of either OX1R inhibitor SB334867 or OX2R inhibitor JNJ10397049 significantly reversed the arousal and neurological benefits of HBO treatment (Figure 2 and 3).

In rats with ketamine induced unconsciousness (Figure 2)—1) There was significance in LORR duration among all the groups, $F(4, 35)=30.73$, $n=40$, $p<0.001$. The duration of LORR was significantly shorter in groups of Ketamine+HBO (49.63 ± 5.93 min, $p<0.001$ vs Ketamine+Air), Ketamine+HBO+vehicle (48.38 ± 5.95 min, $p<0.001$ vs Ketamine+Air) than that in Ketamine+Air group (80.25 ± 5.06 min). There was longer LORR duration in groups of orexin receptor inhibitors pretreated rats. LORR duration in the Ketamine+HBO+SB334867 group was 69.50 ± 9.91 min and Ketamine+HBO+JNJ10397049 was 67 ± 8.25 min, both were significantly different from Ketamine+HBO group ($p<0.001$) and Ketamine+HBO+vehicle ($p<0.001$), respectively.

2) There was significance in the Garcia neurological deficit scores among all the groups, $F(4, 35)=24.02$, $n=40$, $p<0.001$. The Garcia scores were significantly higher in groups of Ketamine+HBO (13 ± 1 , $p<0.001$ vs Ketamine+Air), Ketamine+HBO+vehicle (12 ± 1 , $p<0.001$ vs Ketamine+Air) than that in Ketamine+Air group (9 ± 1). There were lower Garcia scores in groups of orexin receptor inhibitors pretreated rats. Garcia scores in the Ketamine+HBO+SB334867 group was 9 ± 1 and Ketamine+HBO+JNJ10397049 was 9 ± 1 , both were significantly different from the Ketamine+HBO group ($p<0.001$) and the Ketamine+HBO+vehicle ($p<0.001$), respectively.

In rats with ethanol 3.5g/kg induced unconsciousness (Figure 3)—1) There was significance in LORR duration among all the groups, $F(4, 35)=20.03$, $n=40$, $p<0.001$. The duration of LORR was significantly shorter in groups of Ethanol+HBO (65.50 ± 9.59 min, $p<0.001$ vs Ethanol+Air), Ethanol+HBO+vehicle (67.75 ± 10.33 min, $p<0.001$ vs Ethanol+Air) than that in Ethanol+Air group (108.5 ± 12.46 min). There was longer LORR duration in groups of orexin receptor inhibitors pretreated rats. LORR duration in the Ethanol+HBO+SB334867 group was 96.0 ± 14.52 min and the Ethanol+HBO+JNJ10397049 was 94.13 ± 12.10 min, both were significantly different from the Ethanol+HBO group ($p<0.001$) and the Ethanol+HBO+vehicle ($p<0.001$), respectively.

2) There was significance in Garcia neurological deficits scores among all the groups, $F(4, 35)=28.40$, $n=40$, $p<0.001$. The Garcia score was significantly higher in groups of Ethanol+HBO (13 ± 1 , $p<0.001$ vs Ethanol+Air), Ethanol+HBO+vehicle (12 ± 1 , $p<0.001$ vs Ethanol+Air) than that in Ethanol+Air group (9 ± 1). There were lower Garcia scores in groups of orexin receptor inhibitors pretreated rats. Garcia scores in Ethanol+HBO+SB334867 group was 9 ± 1 and Ethanol+HBO+JNJ10397049 was 9 ± 1 , both were significantly different from Ethanol+HBO group ($p<0.001$) and Ethanol+HBO+vehicle ($p<0.001$), respectively.

In rats with ethanol 4.0g/kg induced unconsciousness—Ethanol at 4.0g/kg was associated with a more severe degree of unconsciousness (LORR= 148 ± 20 s) than that of 3.5g/kg (LORR= 107 ± 16 s, $p=0.003$). The arousal benefit of HBO including decreasing LORR and improving Garcia scores were consistently observed in rats with this severe degree of unconsciousness (Data not shown).

HBO Increased the Level of ATP and Orexin A

Six hours after unconsciousness induced by ketamine (100mg/kg) or ethanol (3.5g/kg), the levels of ATP of the somatosensory cortex and hypothalamus significantly increased in HBO

treated rats when compared to Air group (Figure 4). HBO enhanced the expression of orexin A in the somatosensory cortex and hypothalamus areas (Figure 5). The somatosensory cortex and hypothalamus orexin A levels correlated well with LORR in HBO treated rats with unconsciousness induced either by ketamine (Figure 6A) or ethanol (Figure 6B).

ATP level (Figure 4)—1) There was significance in ATP expression within the somatosensory cortex region among all the groups in ketamine (100mg/kg) experiment, $F(3, 20)=15.69$, $n=24$, $p<0.001$. The HBO treatment significantly increased the ATP level of Ketamine+HBO group ($71.67\pm7.76 \mu\text{mol/g}$) than the Ketamine+Air group ($49.17\pm5.85 \mu\text{mol/g}$, $p<0.001$) within somatosensory cortex. ATP level in Ketamine+HBO+SB334867 group was $66.33\pm5.61 \mu\text{mol/g}$ and Ketamine+HBO+JNJ10397049 was $68.83\pm5.56 \mu\text{mol/g}$, both were not different from_Ketamine+HBO group ($p>0.05$), respectively.

2) There was significance in ATP expression within the somatosensory cortex region among all the groups in ethanol (3.5g/kg) experiment, $F(3, 20)=14.10$, $n=24$, $p<0.001$. The HBO treatment significantly increased the ATP level of the Ethanol+HBO group ($68.33\pm8.17 \mu\text{mol/g}$) compared to the Ethanol+Air group ($44.50\pm5.36 \mu\text{mol/g}$, $p<0.001$) within somatosensory cortex. The ATP level in the Ethanol+HBO+SB334867 group was $62.33\pm6.74 \mu\text{mol/g}$ and Ethanol+HBO+JNJ10397049 was $63.83\pm6.79 \mu\text{mol/g}$, both were not different from the_Ethanol+HBO group ($p>0.05$), respectively.

3) There was significance in ATP expression within the hypothalamus region among all the groups in ketamine experiment (100mg/kg), $F(3, 20)=17.91$, $n=24$, $p<0.001$. HBO treatment significantly increased the ATP level of the Ketamine+HBO group ($80.17\pm9.04 \mu\text{mol/g}$) compared to the Ketamine+Air group ($53.17\pm6.55 \mu\text{mol/g}$, $p<0.001$) within somatosensory cortex. The ATP level in the Ketamine+HBO+SB334867 group was $73.50\pm5.01 \mu\text{mol/g}$ and the Ketamine+HBO+JNJ10397049 was $76.00\pm6.60 \mu\text{mol/g}$, both were not different from the Ketamine+HBO group ($p>0.05$), respectively.

4) There was significance in the ATP expression within the hypothalamus region among all the groups in ethanol experiment (3.5g/kg), $F(3, 20)=24.24$, $n=24$, $p<0.001$. The HBO treatment significantly increased the ATP level of the Ethanol+HBO group ($74.83\pm7.63 \mu\text{mol/g}$) compare to the Ethanol+Air group ($47.50\pm5.21 \mu\text{mol/g}$, $p<0.001$) within the somatosensory cortex. The ATP level in the Ethanol+HBO+SB334867 group was $67.67\pm5.24 \mu\text{mol/g}$ and the Ethanol+HBO+JNJ10397049 group was $70.83\pm5.81 \mu\text{mol/g}$, both were not different from Ethanol+HBO group ($p>0.05$), respectively.

Orexin A level (Figure 5)—1) There was significance in orexin A expression within the somatosensory cortex region among all the groups in ketamine experiment (100mg/kg), $F(3, 20)=13.06$, $n=24$, $p<0.001$. The HBO treatment significantly increased the orexin A level of the Ketamine+HBO group ($1.37\pm0.08 \text{ pg/mg}$) compared to the Ketamine+Air group ($1.02\pm0.06 \text{ pg/mg}$, $p<0.001$) within the somatosensory cortex ($p<0.001$). The Orexin A level in the Ketamine+HBO+SB334867 group was $1.29\pm0.09 \text{ pg/mg}$ and the Ketamine+HBO+JNJ10397049 was $1.32\pm0.10 \text{ pg/mg}$, both were not different from the_Ketamine+HBO group ($p>0.05$), respectively.

2) There was significance in the orexin A expression within the somatosensory cortex region among all the groups in ethanol experiment (3.5mg/kg), $F(3, 20)=21.89$, $n=24$, $p<0.001$. The HBO treatment significantly increased the orexin A level of Ethanol+HBO group (1.60 ± 0.15 pg/mg) compared to Ethanol+Air group (1.11 ± 0.09 pg/mg, $p<0.001$) within somatosensory cortex. Orexin A level in Ethanol+HBO+SB334867 group was 1.48 ± 0.10 pg/mg and Ethanol+HBO+JNJ10397049 was 1.54 ± 0.11 pg/mg, both were not different from Ethanol+HBO group ($p>0.05$), respectively.

3) There were significance in orexin A expression within hypothalamus region among all the groups in ketamine experiment (100mg/kg), $F(3, 20)=27.54$, $n=24$, $p<0.001$. HBO treatment significantly increased the orexin A level of Ketamine+HBO group (7.72 ± 0.49 pg/mg) compared to the Ketamine+Air group (5.10 ± 0.47 pg/mg, $p<0.001$) within the somatosensory cortex. Orexin A level in Ketamine+HBO+SB334867 group was 7.06 ± 0.46 pg/mg and Ketamine+HBO+JNJ10397049 group was 7.25 ± 0.70 pg/mg, both were no different from the Ketamine+HBO group ($p>0.05$), respectively.

4) There was significance in the orexin A expression within hypothalamus region among all the groups in ethanol experiment (3.5g/kg), $F(3, 20)=25.96$, $n=24$, $p<0.001$. The HBO treatment significantly increased the orexin A level of the Ethanol+HBO group (7.82 ± 0.59 pg/mg) compared to the Ethanol+Air group (5.26 ± 0.54 pg/mg, $p<0.001$) within the somatosensory cortex. The orexin A level in the Ethanol+HBO+SB334867 group was 7.41 ± 0.53 pg/mg and the Ethanol+HBO+JNJ10397049 group was 7.78 ± 0.67 pg/mg, both were no different from the Ethanol+HBO group ($p>0.05$), respectively.

Orexin A and LORR correlation after HBO treatment (Figure 6)—In the HBO treated ketamine-induced unconscious rats, LORR negatively correlated with orexin A level within the somatosensory cortex ($r=-0.743$, $p<0.001$, $n=18$) and the hypothalamus ($r=0.838$, $p<0.001$, $n=18$). Similarly, the HBO treated ethanol-induced unconscious rats, LORR significantly correlated with the orexin A level within the somatosensory cortex ($r=-0.754$, $p<0.001$, $n=18$) and the hypothalamus ($r=-0.647$, $p=0.004$, $n=18$).

DISCUSSION

In the present study, our novel findings are 1) HBO treatment promoted the arousal in the unconscious rats induced by ketamine or ethanol; 2) HBO-induced upregulation of ATP and orexin A levels were associated with the arousal promotion. The orexin A levels negatively correlated with unconscious duration; 3) Administration of SB334867 (OX1R inhibitor) and JNJ10397049 (OX2R inhibitor) abolished the arousal effects of HBO.

HBO, administering pure oxygen at a pressure greater than that of atmosphere, was demonstrated to improve neurological outcomes in the setting of brain injury via the mechanism of cellular metabolism benefit, anti-inflammation, anti-apoptosis and blood-brain-barrier (BBB) preservation (Buckley et al., 2011; Cossu, 2014; Hu et al., 2016; Huang et al., 2018; Huang and Obenaus, 2011; Huang et al., 2016; Ostrowski et al., 2016). Although HBO has been applied as one of the arousal procedures in patients with prolonged coma (Jiang et al., 2004), few study investigated the direct arousal effect of HBO and the

potential underlying mechanism. In the present study, we used ketamine or ethanol to induce unconsciousness in rats. As an anesthetic drug, ketamine induces unconsciousness by antagonizing N-methyl-D-aspartate (NMDA) class of glutamate receptor in the central nervous system (CNS) (Anis et al., 1983). Alcohol at high concentrations suppresses the activity of CNS and results in unconsciousness (Givens, 1997). Previous studies established the rat model of coma induced by alcohol injection (El Yacoubi et al., 2003; Jia et al., 2012), in which electroencephalogram (EEG) delta activity, a marker of slow wave sleep homeostasis was significantly increased concurrently during LORR (Borbely and Achermann, 1999; Jia et al., 2012). Similarly, we found that both ketamine and ethanol resulted in the LORR immediately after injection, indicating the induction of unconscious status. Additionally, Garcia tests revealed acute neurological deficits 6 hours after unconsciousness induction. In clinical settings, emerging evidence has demonstrated the decreased levels of cerebrospinal fluid orexin A in patients with Hashimoto's encephalopathy, hemorrhagic stroke and traumatic brain injury. The low level of orexin A may contribute to the coma status of these patients (Castillo et al., 2004; Rejdak et al., 2005; Ripley et al., 2001). As important as neuropeptides stimulating cortical activation and arousal, exogenous orexins promoted wakefulness in normal sleeping rats when infusing into basal forebrain structures (España et al., 2001). In anesthetized rats, endogenous orexins play an essential role in facilitating the emergency from general anesthesia (Kelz et al., 2008). Intracerebroventricular (i.c.v.) or intracerebral injection of orexin A in anesthetized rats (Kelz et al., 2008; Shirasaka et al., 2011) significantly induced EEG arousal along with a shortened anesthesia time of ketamine, fluranes, propofol or barbiturates. Orexin A was more potent than orexin B in enhancing neuronal activity through OX1R in the basal forebrain (Dong et al., 2006). Using a rat model of alcohol intoxication-induced coma, Jia et al reported the i.c.v injection of orexin A was able to exert an arousal effect by promoting brain cortical activity through activations of histaminergic, noradrenergic and serotonergic system (Jia et al., 2012). Consistently, we found that there was a significantly higher level of brain orexin A in HBO treated unconscious rats either induced by ethanol or ketamine. Brain orexin A levels were negatively correlated with LORR in HBO treated unconscious rats. These results suggested that the up-regulation of orexin A underlying HBO provided arousal, leading to the neurological benefits. The actions of orexins are mediated by two G protein-coupled receptors, namely, OX1R and OX2R (Ohno and Sakurai, 2008; Sakurai, 2002). In the central nervous system, orexin receptors mainly expressed on neurons (Kukkonen, 2017). While the locus coeruleus (LC) neuron exclusively express OX1R, the tuberomammillary nucleus (TMN) neurons exclusively express OX2R (Ohno and Sakurai, 2008). Orexin receptors antagonists have been clearly shown to promote sleep in animals, which bearing the potential to improve insomnia in patients (Scammell and Winrow, 2011). In our study, either OX1R antagonist or OX2R antagonist could significantly reverse HBO-provided arousal promotion, most likely through blocking the downstream effects of orexin A in the brain, but may not necessary reduce HBO enhanced orexin A production. The results echoed previous findings that the arousal effects of exogenous orexins were blocked by orexin receptor antagonists (Dong et al., 2009; Jia et al., 2012; Kelz et al., 2008; Shirasaka et al., 2011). Given the importance of orexin receptors in arousal signaling, we postulated that pretreatment with either OX1R or OX2R antagonist in the absence of HBO treatment might exacerbate the duration of LORR in rats. Instead, administration of OX1R/

OX2R agonists may result in similar arousal acceleration in the unconsciousness condition. Indeed, recent study demonstrated that administration of an OX2R-selective agonist via i.p. and i.c.v. routes suppressed cataplexy-like episodes in orexin knockout and orexin neuron-ablated mice (Irukayama-Tomobe et al., 2017).

In healthy rats, four weeks of daily HBO treatment (3 ATA for 2h/day) has been shown to significantly increase ATP levels in striated muscle tissues with normal mitochondria (Kurt et al., 2008). One hour of HBO treatment improved cerebral ATP levels after traumatic brain injury in rats, leading to improved cognitive function (Zhou et al., 2007). In our study, we observed the upregulation of ATP by HBO in unconsciousness rats within 6 hours. These cellular bioenergetics effects of HBO could be through regulating mitochondrial function. In normal mice subjected to a single HBO treatment at 2.8 ATA for 90 minutes, a pilot study showed that mitochondrial mass was significantly increased, accompanying by altered the basal respiration and spare respirator capacity in primary cells. Interestingly, in vitro study demonstrated that a disruption of ATP production or reduction in glucose level directly resulted in an inhibition of activity of hypocretin/orexin-containing neurons (Liu et al., 2011). This correlation suggests that the intracellular ATP levels may positively correlated with arousal/wakefulness status (Gao and Horvath, 2014). In the present study, there were significantly increased orexin A accompanying the increased ATP levels within the rat brains. These findings support our hypothesis that the elevation ATP by HBO treatment in orexin neurons enhanced the orexin A synthesis, leading to significant shorter duration of unconsciousness and lesser acute neurological deficits after recovering from ketamine or ethanol-induced unconsciousness. In the rat model of TBI-induced coma, median nerve stimulation upregulated orexin-A and OX1R in the hypothalamic region, leading to a restored righting reflex and response to pain stimuli (Zhong et al., 2015). However, we did not measured the changes in OX1R and OX2R following HBO treatment.

There were some limitations in the present study. Firstly, given that HBO has been shown to be neuroprotective by activating multiple signaling pathways (Hu et al., 2016; Huang et al., 2018; Huang and Obenaus, 2011; Huang et al., 2016; Ostrowski et al., 2016), the possible involvement of other pathways on arousal functions need to be further investigated. Secondly, we used male rats in the current study, which is one of the limitations. Female rats have been reported to have higher levels of baseline levels of prepro-orexin mRNA (Johren et al., 2002), increased cerebrospinal fluid orexin A expression and higher activation of orexin-producing neurons compared with control males (Grafe et al., 2017). Additionally, the hormone cycle may have the effect on orexin A in female rats. During menopause, the lower estrogen levels were associated with an increased orexin A levels (Messina et al., 2013). Those gender differences in orexin levels may require a separate study to establish unconsciousness model in female rats as well as apply a different HBO regimen for arousal. Thus further study is warranted to validate the HBO arousal effect and orexin A mechanism in female rats.

CONCLUSION

HBO was able to promote arousal via at least in part up-regulation ATP/orexin A in unconscious rats induced by ketamine or ethanol. Our results suggest that HBO may be a practical clinical approach to accelerate coma recovery in patients.

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SIGNIFICANCE STATEMENT

Approaches that specifically facilitate the recovery from coma would have enormous impacts on patient outcomes and medical economics. Orexin-producing neurons release orexin neuropeptides (also known as hypocretins) energy-dependently to regulate the vigilance states. Hyperbaric oxygen was able to promote arousal in unconscious rats, at least in part, by up-regulation energy production and orexins neuropeptides level in the brain. Thus, HBO may offer a practical clinical approach to accelerate coma recovery in patients.

Experiment 1: Ketamine

Ketamine+Air
Ketamine+HBO
Ketamine+HBO+Vehicle(DMSO)
Ketamine+HBO+SB334867
Ketamine+HBO+JNJ10397049

Experiment 2: Ethanol

Ethanol+Air
Ethanol+HBO
Ethanol+HBO+Vehicle (DMSO)
Ethanol+HBO+SB334867
Ethanol+HBO+JNJ10397049

Figure 1.

Experimental design and animal group classification. HBO=Hyperbaric Oxygen; LORR= Duration of loss of righting reflex; DMSO= Dimethyl sulfoxide.

Figure 2.

Arousal effects of HBO on the duration of loss of righting reflex (LORR) and Garcia neurological scores in ketamine-induced unconscious rats. (A) HBO significantly shortened the duration of LORR induced by ketamine. (B) HBO significantly improved the acute neurologic deficits in the rats 6 hours after the unconsciousness induction. SB334867 and JNJ10397049 significantly reversed the arousal and neuroprotective effects of HBO by blocking OX1R and OX2R, respectively. $n=8/\text{group}$, $*P<0.001$ versus Air, $\#P<0.001$ versus HBO.

Figure 3.

Arousal effects of HBO on the duration of LORR and Garcia neurological scores in ethanol-induced unconscious rats. (A) HBO significantly shortened the duration of LORR induced by 3.5g/kg ethanol. (B) HBO significantly improved the acute neurologic deficits in the rats 6 hours after the unconsciousness induction. SB334867 and JNJ10397049 significantly reversed the arousal and neuroprotective effects of HBO by blocking OX1R and OX2R, respectively. n=8/group, * $P<0.001$ versus Air, # $P<0.001$ versus HBO.

Figure 4.

ELISA assay showed that HBO treatment significantly increased the ATP level within the somatosensory cortex and hypothalamus regions 6 hour after unconsciousness induced either by ketamine at 100mg/kg (A) or ethanol at 3.5g/kg (B). Orexin receptors inhibition by SB334867 or JNJ10397049 did not affect HBO-induced upregulation of ATP level. n=6/group, * $P < 0.001$ versus Air.

Figure 5.

ELISA assay showed that HBO treatment significantly increased the orexin A levels within the somatosensory cortex and hypothalamus regions 6 hour after unconsciousness induced either by ketamine at 100mg/kg (A) or ethanol at 3.5k/kg (B). Orexin receptors inhibition by SB334867 or JNJ10397049 did not affect HBO-induced upregulation of orexin A level. n=6/group, * $P<0.001$ versus Air.

A. Ketamine

B. Ethanol

Figure 6.

The orexin A levels within the somatosensory cortex and hypothalamus regions were significantly correlated negatively with LORR in the HBO treated unconscious rats either induced by ketamine at 100mg/kg (A) or ethanol at 3.5g/kg (B).