Journal of Biosciences and Medicines, 2024, 12, 304-315
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Effects of Intermittent Hypoxia Exposure on Symptoms of Chronic Fatigue Syndrome in Repeated Restraint Stress and Forced Swimming Induced-Mouse Model

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How to cite this paper: Mabounda Kounga, P.R., Zhang, Y.J., Wang, H.X., Wang, R. and Chen, P.J. (2024) Effects of Intermittent Hypoxia Exposure on Symptoms of Chronic Fatigue Syndrome in Repeated Restraint Stress and Forced Swimming Induced-Mouse Model. *Journal of Biosciences and Medicines*, **12**, 304-315.
https://doi.org/10.4236/jbm.2024.1210026

Received: September 11, 2024
Accepted: October 21, 2024
Published: October 24, 2024

Abstract

Background: Chronic fatigue syndrome (CFS) shows as its main symptoms debilitating fatigue that is not relieved by physiological rest, depression, inflammation, learning disability and memory impairment. But, intermittent hypoxia, consisting of alternating exposure to hypoxia and normoxia, plays a very important role in improving CFS. However, the essential components for improving learning and memory in CFS patients as well as their mechanism are largely unknown. **Objectives:** This study aims to analyze the effects of 12% and 15% hypoxia on the expression of alpha tumor necrosis factor (TNF-α) and nuclear factor kappa B (NF-κB) in CFS induced-mouse model for clarifying the effects on the learning and memory function. **Methods**: A total of 48 type IC mice were used. The CFS mouse model was established using restrained stress and repeated forced swimming. Treatment of CFS was done by exposing CFS mice to intermittent hypoxia at 12% and 15%. The effects of intermittent hypoxia on learning and memory as well as its mechanism of action on inflammation were tested respectively with the Morris test, the SDS page, the immunohistochemistry technique and the Nissl staining. **Results**: We found that 12% and 15% intermittent hypoxia exposure improved learning capacity and memory of CFS induced-mice. SDS page showed that CFS caused higher TNF-α expression. By exposing CFS mice to 12% and 15% intermittent hypoxia, TNF-α expression decreased significantly, with a much better effect at 15%. Both TNF-α and NF-κB increased in CFS state and decreased after treatment with intermittent hypoxia. **Conclusion**: Intermittent hypoxia improves learning capacity and memory. It acted by decreasing NF-κB come to down-regulating TNF-α and ameliorates learning capacity and

memory impairment in CFS mice.

Keywords

Chronic Fatigue Syndrome, Intermittent Hypoxia, Stress, Learning Capacity, Memory

1. Introduction

Chronic fatigue syndrome (CFS) is a learning and memory disorder caused by the interaction of social and psychological factors [1] [2]. Studies have shown that chronic stress has a great impact on animals and the human hippocampus, mainly reflected in the neuroendocrine-immune network, the body on the regulation of chronic stress disorders, and ultimately induces chronic inflammation, hippocampal spinal atrophy, synaptic changes, neurogenic functional damage, in which decline in learning and memory function as the main index [2]-[5]. In other words, learning et memory impairment in CFS patients could be explained by the effects of psychiatric disorders or the combination of a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis [3] [4].

Nowadays, it is believed that the decrease in learning and memory function in CFS patients is mainly due to the interaction of pro-inflammatory factors such as Interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and Interleukin-1α (IL-1α), as well as reactive oxygen species (ROS), nuclear factor-kappa B (NF-κB) and cortisol [3] [5]-[7].

TNF-α and its signaling pathway, notably nuclear factor kappa B (NF-κB), is a key molecule in the initiation of the inflammatory cascade which is extremely reactive to stress [1]. It plays a very important role in the regulation of messenger ribonucleic acids (mRNA) of cytokines like IL-6 and IL-1, leading to an inflammatory response in the brain [8]-[10]. It also regulates genes related to immune inflammation through NF-κB, causing atrophy and apoptosis of hippocampal pyramidal cells and decline in learning and memory [11]. The expression of NF-κB in CFS patients was significantly increased [12]-[15], which may be related to the decline in learning and memory ability. Therefore, it seems that inhibition of the level of inflammatory factors and abnormal expression of TNF-α and NF-κB is helpful in improving learning and memory in CFS patients.

It has been reported that intermittent hypoxic exposure appears to improve anti-inflammatory status. Indeed, a period of hypoxia followed by normoxia induces more reactive oxygen species (ROS) triggering a redox signaling cascade and the synthesis of protective intracellular proteins such as antioxidant enzymes and heat shock proteins [16]. But, the physiological function of hypoxia exposure and its role in regulating immune function and health mediation is very complex. When cells are exposed to high-intensity hypoxia, they feel in an inappropriate state. In contrast, prolonged exposure to intermittent hypoxia promotes cellular tolerance and reduced

secretion of pro-inflammatory cytokines [17]-[19]. This action of protecting neurons promotes the regeneration of nerve cells, the synaptic plasticity of hippocampal neurons and improves learning and memory capacity [17] [19]. The beneficial effects of intermittent hypoxic protocols are well described in numerous studies. The effectiveness of hypoxic exposure has been studied for the treatment and rehabilitation of patients suffering from obesity, systemic hypertension, type 2 diabetes and bronchial asthma [16] [20]-[22]. We showed that exposure to 15 and 18% hypoxia improved serum levels of IL-6, TNF-α, and IL-1β in mice with fatigue syndrome, as well as the ability to learn and memory [23]. Therefore, the effects of different concentrations of hypoxia "12% and 15%" vs "normoxia" on the expression of pro-inflammatory factor TNF-α and NF-κB in the hippocampus of CFS induced-mouse model were selected to clarify the effects of intermittent hypoxia exposure on the learning and memory function of CFS model mice.

2. Methodology

2.1. Animals Groups

The Institutional Animal Care and Use Committee of Shanghai University of Sport (China) had approved our animal protocol. All animal studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals revised in 2011.

A total of 48 IC type mice, 30 to 40 g of specific pathogen free (SPF) were selected as the animal population. They housed 6 mice per cage and the environmental temperature and humidity were 23˚C ± 2˚C and 55 ± 15%, respectively. They were provided by Shanghai Sipprbk laboratory animal company (No. SCXK2008-0016). All mice had standard 12 h light cycle with light from 6 AM to 18 PM. They were fed a regular mouse diet provided by the Changhai Hospital laboratory for mice. They were housed in regular mouse cages of about 30 cm x 20 cm × 16 cm. After adapting to the environment of our laboratory for 4 days, they were randomly assigned to four groups: a control group (C), a chronic fatigue syndrome group (SFC), a treatment to 12% hypoxia exposure group (12%-SFC), and a treatment to 15% hypoxia exposure group (15%-SFC).

2.2. The Creation of CFS and Treatment

The mice into SFC, 12%-SFC , and 15%-SFC groups were restrained in ventilated tubes (50 mL) and forced swimming in a plastic box (40 width × 75 length × 40 depths) using a protocol development in our laboratory [23]. In summary, during week 2, 3, 4 and 5 the time of restrained stress was gradually increased to 3 h/day, 6 h/day, 10 h/day and 9 h/day respectively. Just after, they were forced to swim from 1 hour, 1 hour 30 minutes until exhausted and 1 hour 30 minutes during week 2, 3, 4 and 5 respectively. After the establishment of the CFS induced-model mice, the 12%-SFC and 15%-SFC groups were exposed to 12 and 15% intermittent hypoxia respectively for 4 hours/day during 10 days.

2.3. Morris Water Maze Test

The Morris water maze test was performed the last 6 days of intermittent hypoxia exposure (5^{th} to 10^{th} day). The apparatus consisted of a circular water pool 100 cm in diameter and 40 cm in height. It was filled with 23˚C ± 1˚C water with a depth of 40 cm and covered a black platform (10 cm in diameter). The platform was submerged approximately 1.5 cm below the surface of water. The pool was divided into four quadrants: northeast (NE), northwest (NW), southeast (SE), and southwest (SW) at equal distances on the rim. The platform was located in the center of southwest quadrant. During the first 3 days acquisition test, mice were given 8 trials per day to find the hidden platform. Each mouse (6 mice per group) was gently placed into the water facing the wall in the direction of north (N), east (E), south (S), and west (W) in series of order reported by Vorhees [24]. The mouse was allowed to swim until they reached the hidden platform (maximum swim time was 120 seconds). The escape latency to reach the platform was recorded and they remained on the platform for 20 seconds before being removed. The mouse which failed to find the platform within 120 seconds was guided to the hidden platform and then was placed on the platform for 15 seconds for reinforcement before being removed.

One trial of the retention test without the platform was performed on the 4th day to assess the memory of the correct platform location. The mice were placed into the pool and swam freely for 60 seconds. The swimming paths were recorded by a video camera linked to a computer-based image analyzer (Anhui Huaibei Zhenghua Instrument Biologique et Équipement Co., Ltd., China). The number of target heading and the swimming time in each zone were analyzed. The mice were sacrificed 72 hours to avoid stress influence and the brains were randomly used either for western blotting (6 mice) or immunohistochemistry (6 mice).

2.4. Western Blotting

At 72 hours after he Morris water maze test, the mice were deeply anesthetized and the hippocampal tissue was rapidly dissected. The hippocampal tissue was homogenized and sonicated on ice in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1% protease inhibitor cocktail; Sigma). After centrifugation, the supernatant was collected and assayed for protein concentration using the Bradford method. Lysate samples containing 100 μL of protein were fractionated by SDS-10% polyacrylamide gel electrophoresis, and then subjected to Western blot analysis.

2.5. Immunohistochemistry

After the retention test trial, the mice were deeply anesthetized and the brain was removed and post fixed in the same perfusing solution overnight at 4˚C. 20 μm thick coronal sections of brain tissue were made using a freezing microtome (Leica, 1900 CM, Germany). The brain sections were stained by the free-floating DAB reaction. The sections were rinsed with 0.05 M PBS and incubated for 15 min in 1% hydrogen peroxide PBS at room temperature. The sections were

incubated overnight at 4˚C with primary antibody against TNF-α (1:100, American Cell Signaling Technology Co., Ltd.) and mouse monoclonal antibodies against the p65 subunit (1:500, American Santa Cruz Biotechnology Co., Ltd.), then incubated with polyclonal anti-rabbit IgG secondary antibody (1:500, Shanghai Wei'ao biotechnology Co., Ltd.) for 1 hour at room temperature, after which the avidin-biotin complex (Wuhan BOSHIDE Biotechnology Co., Ltd.) method was carried out with peroxidase coupling in a mixture containing 0.05% DAB (Shanghai Biotin Co., Ltd.) and 0.03% H_2O_2 for 2 - 5 minutes. Images of the DAB-colorized brain sections were captured using a light microscope (BX51, Olympus, Japan) equipped.

2.6. Nissl Staining

Histological examinations were performed using an optical microscope (Nikon, Japan) after Nissl staining. For Nissl staining, 5-μm of coronal sections was first prepared. These sections were deparaffinized in xylene and then hydrated. After rinsing with tap water and then distilled water, sections were stained with 0.1% cresyl violet for 3 min. After dehydration, samples were finally placed under a glass coverslip. The Nissl body was dyed purple-blue.

2.7. Statistical Analysis

All data were expressed as mean ± standard deviation, evacuation latency was analyzed by repeated variance analysis, and other indices were processed by one way analyze of variance (ANOVA). All data processing was completed by using SPSS 25.0 statistical software, in which $p < 0.05$ was a significant difference.

3. Results

3.1. Influence of Exposure to 12% and 15% Intermittent Hypoxia for 10 Days on the Weight of CFS Induced-Mice

Table 1 shows that 12% and 15% intermittent hypoxia exposure for 10 days greatly influenced the weights of the mice. The body weight of mice in groups SFC, 12%-SFC and 15%-SFC was significantly lower than that in group C ($p < 0.05$). During exposure to hypoxia on the 2nd, 6th and 10th days, the body weights of mice in the 12%-SFC group were significantly lower compared to that of mice in the SFC group ($p < 0.05$), but there was no had no significant difference compared to the 15%-SFC group ($p > 0.05$); compared to the 12%-SFC and SFC groups, there is no significant difference in weight for the 12%-SFC group ($p > 0.05$).

Table 1. Influence of exposure to 12% and 15% intermittent hypoxia on the weight of SFC-induced mice.

Days	C	CFS	12%-CFS	15%-CFS
2nd day	34.50 ± 0.75	28.04 ± 0.32*	26.67 ± 0.39*#	27.47 ± 0.39*
4th day	34.67 ± 0.75	28.70 ± 0.29*	27.50 ± 0.41*	28.11 ± 0.36*
6th day	33.94 ± 0.67	28.98 ± 0.34*	27.62 ± 0.49*#	28.46 ± 0.38*

Continued

8th day	34.04 ± 0.76	30.01 ± 0.38*	28.37 ± 1.97*#	30.02 ± 0.36*
10th day	34.57 ± 0.73	30.91 ± 0.53*	29.67 ± 0.40*#	30.51 ± 0.46*

*p < 0.05 compared to C group; #p < 0.05 compared to CFS group.

3.2. Effects of Intermittent Hypoxia Exposure for 10 Days (4 h/day) on Spatial Learning and Memory Capacity

Spatial learning (Figure 1) was assessed as a function of training day with respect to the following parameters: (A) escape latency, (B) percentage of time spent in the target quadrant. (A) The escape shows the changes obtained after 12% and 15% intermittent hypoxia exposure for 10 days (4 hours/day) over the latency time. The repeated measures ANOVA showed a main effect of F (3.37) = 0.724, p > 0.05. It appears that there is no significant difference between the different groups. One-way ANOVA performed on the first, second, third, fourth and fifth day of exposure to hypoxia did not present a different latency between the different groups (p > 0.05). (B) In the target quadrant. The one-way ANOVA shows that there is no significant difference between the groups C, CFS, 12%-CFS and 15%-CFS regarding the time spent in the target quadrant (p > 0 .05).

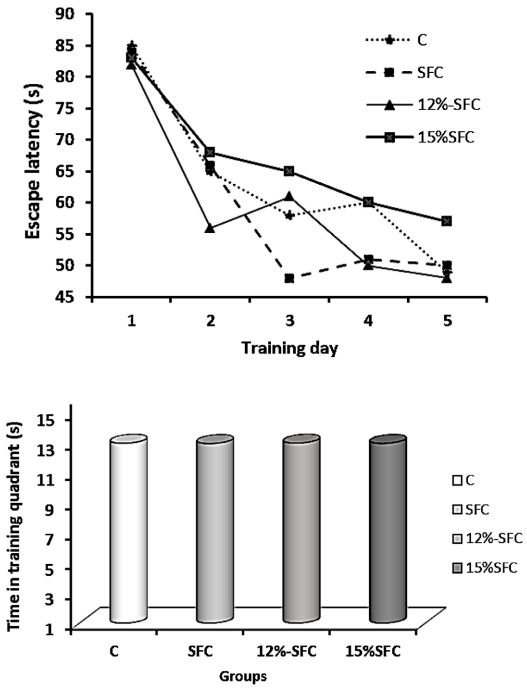


Figure 1. Latency time of CFS mice exposed to 12% and 15% intermittent hypoxia for 10 days.

3.3. Effects of Restraint Stress and Repeated Forced Swimming on Neuronal Damage as Well as Intermittent Hypoxia Exposure in the CA3 of the Hippocampus

Representative photographs (Figure 2) show CA3 neurons labeled cells in the

hippocampus of the mice which performed the Morris water maze test. The Nissl staining results show that 15% exposure improved the degree of disorganization of the hippocampal structure. Compared to 12%, the improvement observed in the hippocampi of mice in the 15%-CFS groups was better.

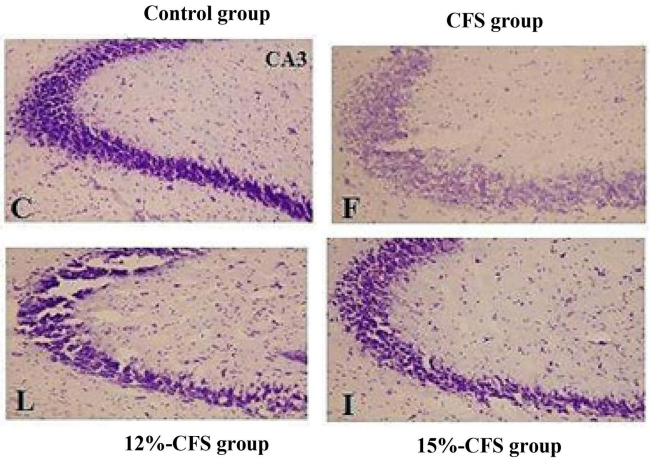
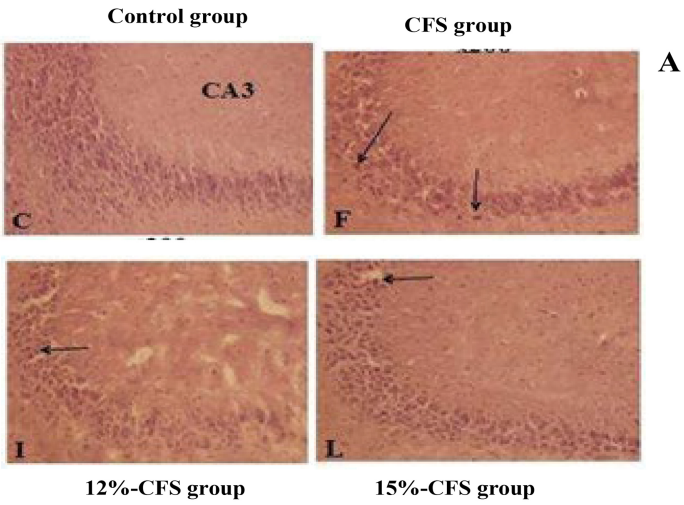


Figure 2. Effects of CFS and 12% with 15% intermittent hypoxia exposure in hippocampal of CFS-induced mice.

3.4. Effects of Restraint Stress and Repeated Forced Swimming on Neuronal Damage as Well as Intermittent Hypoxia Exposure in the CA3 of the Hippocampus

Figure 3(A) shows representative of the TNF-*α* positive expression in the hippocampus. In the SFC group, TNF-*α* expression was much higher than that of the C group. Compared to CFS group, TNF-*α* expression decreased significantly in the hippocampus 12% and 15% intermittent hypoxia exposure while the effect of the 15%-CFS group was better. In Figure 3(B), representative photographs show CA3 neurons labeled cells in the hippocampus of the mice which performed the Morris water maze test.



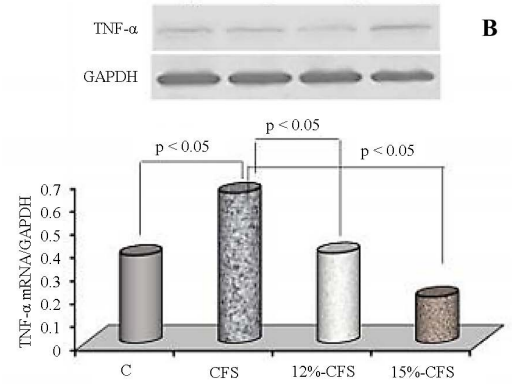


Figure 3. TNF-*α* and NF-*κ*B localization, TNF-*α* expression and effects of 12% with 15% intermittent hypoxia exposure in hippocampal of CFS mice.

4. Discussion

The results showed that restraint stress and forced swimming for five weeks induced CFS mice. CFS leads to learning and memory impairments along with increased TNF-*α* expression in hippocampus. When exposed to 12% and 15% hypoxia, the spatial learning and memory were improved and TNF-*α* expression decreased. After the CFS model was established, TNF-*α* and NF-*κ*B were actively localized in the hippocampus. After exposure to intermittent hypoxia, TNF-*α* and NF-*κ*B were less localized.

The learning and memory impairments of CFS-induced mice [25] correspond to symptoms seen in CFS patients. It suggests that the effect of modeling CFS and hypoxia treatment can be judged by assessing learning and memory ability. In the present study, it was found that restraint stress and repeated forced swimming significantly prolong the escape latency time of mice. Furthermore, the time spent searching the platform quadrant was significantly shortened in CFS mice, suggesting that both types of stress lead to learning and memory impairments.

We observed that the expression of TNF-*α* significantly increased after restraint and swimming. It is known that low levels of TNF-*α* were involved in hippocampal development, synthesis of brain-derived nutritional factors, maturation of the dentate gyrus, and nerve connections between CA3 and CAI [26] [27]. But any increase in TNF-*α* expression affects the establishment of hippocampal morphology and synaptic connections, and then will impair learning and memory ability. In our study, increased TNF-*α* expression led to chronic inflammation that negatively affected learning and memory. Another explanation is that high levels of TNF-*α*, inhibit nerve regeneration and reduce the synaptic effect of cerebrospinal fluid, leading to a decrease in learning and memory ability [28].

When CFS-induced mice were exposed to 12% and 15% intermittent hypoxia, the latency time was not altered. Similarly, there was no significant difference in the time spent in the target quadrant. After analysis, we observe that intermittent hypoxia significantly decreases the TNF-*α* expression with 12% and 15%

intermittent hypoxia showing similar significant effects. This result is quite similar to that reported by Naghshin *et al.* [29]. Indeed, authors by manipulating of inspired oxygen from 20.9 to 5.0% - 6.0% over 30 seconds conclude that exposure to IH induces adaptive responses in not only healthy animals but also in animals with underlying heart failure. In this study, 15% intermittent hypoxia exposure shows better significant effects. It was found that 12% intermittent hypoxia exposure for 1 h/days, 5 days/week, 4 ≤ 8 weeks significantly reduced antioxidant components, promoted inflammation and oxidative stress mediated cell apoptosis. Whereas, it does not appear in the 15% intermittent hypoxia exposure [30] [31].

Overall, the expression of NF-κB and TNF-α was significantly increased, suggesting that they were involved in impaired learning and memory in CFS patients. Abnormal activated NF-κB may induce the decrease in the synthesis of brain-derived nutritional factors through direct exposure to the protein kinase A (PKA)/cAMP-responsive element binding protein (CREB) pathway, and then affect learning and memory ability. Studies have shown that preventing abnormal ONF activation can improve memory ability [32]. It seems that the mechanism by which CFS induces learning and memory disorders is its action on the increase of TNF-alpha which, in turn, activates NF-κB chronically. It is known that abnormal NF-κB activation is a factor in impaired memory in CFS patients [33] [34]. Indeed, abnormal NF-κB activation not only led to high expression of pro-inflammatory cytokines [35] [36], but also decreased the PKA/CREB pathway affecting a variety of gene expression and protein synthesis [37]. It seems that, 12% and 15% intermittent hypoxia exposure can contribute to the ideal effect. As a therapeutic target of NF-κB, it is of great importance to activate NF-κB, for reducing the expression of TNF-α and improving the PKA/CREB pathway through an intervention. Hypoxia may play a role in regulating microglia function and lead to their anti-inflammation improvement. The amount of 12% and 15% hypoxia concentration could up-regulate microglia activation and improve the treatment of CFS. These moderate exposure concentrations of hypoxia may play an indirect or direct role for increasing cell membrane stability, enhancing synaptic effect, regulating glutamate and its N-methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, promote calcium ionic, Cyclic adenosine monophosphate (cAMP)/PKA pathway, inhibit the inflammatory response and improve learning and memory ability [30].

5. Conclusion

Restraint stress and repeated forced swimming induced CFS in mice. CFS significantly led to learning and memory impairments, increased TNF-α expression and increased the TNF-α and NF-κB activity in the hippocampus. Exposure to 12% and 15% intermittent hypoxia significantly improved learning and memory abilities, reduced the TNF-α expression and increased the TNF-α and NF-κB activity. Intermittent hypoxia mediates the reduction level the TNF-α expression and chronic inflammation by down-regulating the NF-κB.

Conflicts of Interest

The authors declare no conflicts of interest.

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