科技部補助專題研究計畫報告

高壓氧治療SCA17小鼠之持久性、再現性、強度與分子效應

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本研究具有政策應用參考價值：■否 □是，建議提供機關
（勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
本研究具影響公共利益之重大發現：□否 □是

中 華 民 國 110 年 01 月 31 日

中 文 摘 要 ： 第17型脊髓小腦共濟失調症（SCA17）是一種顯性遺傳神經退化性疾病，患者有漸進式的小腦萎縮、共濟失調及認知障礙等病徵。目前沒有任何有療效的藥物可以治療SCA17。本研究旨在探討高壓氧治療(HBOT)對SCA17 基因轉殖小鼠行為改善及神經保護的作用。HBOT是一種較為非侵入性的治療方式，目前已用於治療一氧化碳中毒、動脈栓塞、創傷性腦損傷、燒燙傷及減壓病(潛水夫病)等；已有文獻證實，HBOT可以降低發炎反應的發生、改善組織缺氧的狀態，並促使微血管增生。本研究採用SCA17基因轉殖小鼠，進行各式行為實驗(曠野實驗、Y字迷宮、步態分析及滾輪測試)評估其運動和空間記憶的能力；經過HBOT，我們發現SCA17 TG小鼠的焦慮情況、短期記憶及運動協調能力有顯著改善。我們也以每個月進行一次行為實驗之方式得知HBOT效果的持續性可以維持2-3個月。在小腦組織病理切片結果中，我們發現，HBOT降低了SCA17 TG小鼠小腦中Purkinje cell丟失，使其排列順序變整齊、細胞本體較為飽滿及神經纖維密度上升；也發現HBOT能夠減緩SCA17 TG小鼠神經發炎反應之astrocyte及microglia；另外，與記憶相關及細胞增殖之蛋白路徑AKT/CaMKII-ERK-CREB在SCA17小腦中之蛋白活化也在HBOT後顯著上升。因此，我們認為HBOT對於SCA17具有治療的發展潛力。

中 文 關 鍵 詞 ： 第十七型脊髓小腦運動失調症, 基因轉殖小鼠, 高壓氧治療

英 文 摘 要 ： We have established SCA17 transgenic mouse model which shows phenotypes similar to the clinical patients and could be used to evaluate potential therapeutic treatments for SCA17. Hyperbaric oxygen treatment (HBOT) is considered to be a less invasive therapy for many conditions. Our previous studies indicate that oxidative stress, neuroinflammation and neuronal apoptosis are elevated in the SCA17 mice, which are the main targets of HBOT. Therefore, we expect HBOT to be a therapeutic option for the management of SCA17. We first Identified the lasting effect of HBOT (2.2 ATA for 14 days) on SCA17 mice. Behavior tasks were conducted on the mice before HBO, right after HBOT and every month thereafter to examine how long the beneficial effect could be lasted in these mice. We then investigated whether the beneficial effect of one more cycle of HBOT in HBO-treated mice. The protein quantitative and histopathological staining analyses show that HBOT can promote CMA, reduce PC death, and inhibit nerve inflammation in SCA17 TG mice, which further improve cognition. In addition, oxidative stress test, complete blood count test, blood biochemistry and the results of liver, and kidney pathological examination indicated that HBOT is a safe treatment. HBOT could be potential to be developed into an alternative or therapeutic treatment for SCA17. Accumulated experimental findings have revealed the similarity in disease pathomechanisms and possible therapeutic strategies in polyQ diseases, therefore HBO

could be potential alternative or therapeutic treatment for other polyQ diseases caused by chronic genetic mutations other than SCA17.

英文關鍵詞：spinocerebellar ataxias, polyglutamine, SCA17, hyperbaric oxygen

摘要

第17型脊髓小腦共濟失調症 (SCA17) 是一種顯性遺傳神經退化性疾病，患者有漸進式的小腦萎縮、共濟失調及認知障礙等病徵。目前沒有任何有療效的藥物可以治療SCA17。本研究旨在探討高壓氧治療(HBOT)對SCA17 基因轉殖小鼠行為改善及神經保護的作用。HBOT是一種較為非侵入性的治療方式，目前已用於治療一氧化碳中毒、動脈栓塞、創傷性腦損傷、燒燙傷及減壓病(潛水夫病)等；已有文獻證實，HBOT可以降低發炎反應的發生、改善組織缺氧的狀態，並促使微血管增生。本研究採用SCA17基因轉殖小鼠，進行各式行為實驗(曠野實驗、Y字迷宮、步態分析及滾輪測試)評估其運動和空間記憶的能力；經過HBOT，我們發現SCA17 TG小鼠的焦慮情況、短期記憶及運動協調能力有顯著改善。我們也以每個月進行一次行為實驗之方式得知HBOT效果的持續性可以維持2-3個月。在小腦組織病理切片結果中，我們發現，HBOT降低了SCA17 TG小鼠小腦中Purkinje cell丟失，使其排列順序變整齊、細胞本體較為飽滿及神經纖維密度上升；也發現HBOT能夠減緩SCA17 TG小鼠神經發炎反應之astrocyte及microglia；另外，與記憶相關及細胞增殖之蛋白路徑AKT/CaMKII-ERK-CREB在SCA17小腦中之蛋白活化也在HBOT後顯著上升。因此，我們認為HBOT對於SCA17具有治療的發展潛力。

關鍵字：第十七型脊髓小腦運動失調症, 基因轉殖小鼠, 高壓氧治療

Abstract

We have established SCA17 transgenic mouse model which shows phenotypes similar to the clinical patients and could be used to evaluate potential therapeutic treatments for SCA17. Hyperbaric oxygen treatment (HBOT) is considered to be a less invasive therapy for many conditions. Our previous studies indicate that oxidative stress, neuroinflammation and neuronal apoptosis are elevated in the SCA17 mice, which are the main targets of HBOT. Therefore, we expect HBOT to be a therapeutic option for the management of SCA17. We first Identified the lasting effect of HBOT (2.2 ATA for 14 days) on SCA17 mice. Behavior tasks were conducted on the mice before HBO, right after HBOT and every month thereafter to examine how long the beneficial effect could be lasted in these mice. We then investigated whether the beneficial effect of one more cycle of HBOT in HBO-treated mice. The protein quantitative and histopathological staining analyses show that HBOT can promote CMA, reduce PC death, and inhibit nerve inflammation in SCA17 TG mice, which further improve cognition. In addition, oxidative stress test, complete blood count test, blood biochemistry and the results of liver, and kidney pathological examination indicated that HBOT is a safe treatment. HBOT could be potential to be developed into an alternative or therapeutic treatment for SCA17. Accumulated experimental

findings have revealed the similarity in disease pathomechanisms and possible therapeutic strategies in polyQ diseases, therefore HBO could be potential alternative or therapeutic treatment for other polyQ diseases caused by chronic genetic mutations other than SCA17.

Key words: spinocerebellar ataxias, polyglutamine, SCA17, hyperbaric oxygen

Introduction

The dominantly inherited spinocerebellar ataxias (SCAs) are a diverse group of neurodegenerative diseases. The most common SCAs are caused by CAG repeat expansion in the disease-causing genes. The extended CAG encodes a long polyglutamine (polyQ) tract which leads to intracellular accumulation of aggregated proteins and cell death. These polyQ SCAs include types 1, 2, 3, 6, 7, 8, 17 and DRPLA. The symptoms of polyQ SCAs typically start in adulthood and slowly progress for many years and mostly culminate in death by brainstem failure (Paulson et al., 2017). The neurological abnormalities of patients include gait ataxia, limb incoordination, speech disturbance and oculomotor abnormalities (Ashizawa and Xia, 2016). Considerable variability in disease features is partly owing to the size of the polyQ repeat expansion and negatively correlates with the age of onset. Longer polyQ expansions are usually associated with earlier onset and more severe neurological symptoms (Koide et al., 1999; Khare et al., 2005).

SCA17 was resulted from the trinucleotide repeat expansion in the region of a polymorphic CAG/CAA repeat segment of *TBP* gene on chromosome 6q27 (Koide et al., 1999). The range of repeat numbers in normal population is 25-40, while more than 41 repeats has been reported in various familial and sporadic ataxia patients (Buijsen et al., 2019). Adult-onset clinical phenotype includes ataxia, eye movement abnormality, cognitive decline, psychiatric symptoms, dementia, dystonia and parkinsonism (Rolfs et al., 2003; Hagenah et al., 2004; Hubner et al., 2007). To elucidate the pathomechanism and identify potential treatment for SCA17, the SCA17 transgenic mice harboring human TBP-109Q were established in our laboratory and with the pathological features recapitulate the phenotypes of SCA17 patients (Chang et al., 2011; Chang et al., 2016). The SCA17 mice have been used as a disease model to evaluate therapeutic effect of potential treatments for SCA17 (Chen et al., 2019a; Lin et al., 2020).

SCAs are known as relentlessly progressive and fatal diseases. More than 100 clinical trials for SCA treatments are completed or ongoing until January 2020 (Chen et al.,

2020). The tested synthetic compounds include neurotransmitter modulators, ion transport inhibitors, growth factors, histone deacetylase (HDAC) inhibitors, and autophagy enhancers (Chen et al., 2020). Stem cell therapy is also another major strategy conducted in the clinical trial, which adipocyte- and umbilical cord-derived stem cells are two major sources for cell-based treatment (Chen et al., 2020). However, no Food and Drug Administration (FDA) or European Medicines Agency (EMA) approved drug for SCAs until now. It seems no therapies are likely to revolutionize the SCA treatment shortly; therefore, it may be helpful to pursue alternative strategies. Hyperbaric oxygen treatment (HBOT) has been used to treat more than 100 disorders worldwide over the years without marked side effect (Edwards, 2010). So far there is no study ever reports the treatment effect of HBO on SCA diseases, in this study, we intend to evaluate the HBOT effect in SCAs using the SCA17 transgenic mice.

HBOT provides 100% oxygen at a pressure greater than that at sea level, atmospheres absolute pressure (ATA, 1ATA=14.7 psi) and mostly 1.2-3.0 ATA, which results in arterial oxygen tension often exceeds 2,000 mmHg, and 200-400 mmHg in tissues (Fosen and Thom, 2014). It's reported HBOT increases the level of oxygen dissolved in plasma from 0.3 ml/dL to 6 ml/dL at 3ATA (Tibbles and Edelsberg, 1996). HBOT ensures that oxygen required by tissues can be obtained from plasma. As the oxygen is in a solution, it can reach the areas where red blood cells are limited(Gill and Bell, 2004; Atzeni et al., 2020).

HBO has been used to treat more than 100 disorders worldwide over the years despite no sufficient scientific evidence regarding its benefit and safety (Edwards, 2010). It was reported HBO affect the oxygen toxicity in central nervous system and give rise some transient epileptic episodes (Pepper et al., 1992). The side effects result from the HBOT are mostly mild and reversible (Pepper et al., 1992). A study showed that HBO treatment stimulates the expression levels and activity of anti-oxidant enzymes, so the homeostasis and the redox (reductive/oxidative) cell state were maintained, and ensure treatment safety (Thom, 2011). HBOT is still considered to be a less invasive therapy for many conditions, including neurodevelopmental and neurodegenerative disorders (Smallwood and Murray, 1999; Rossignol and Rossignol, 2006). In addition, growing interest in the application of HBO to treat neurodegenerative diseases is because no satisfying results are achieved with currently used pharmacological therapies.

HBO have neuroprotective effects against traumatic/ischemic brains and spinal cord

injury (Hendee, 1989; Huang et al., 2013; Xu et al., 2016). It was documented that HBO increased oxygen supply and improved neural metabolism after ischemia (Calvert et al., 2007). In addition, HBO therapy alleviated impairments associated with strokes, such as memory loss, language, and comprehension deficits (Sun et al., 2014; Zhai et al., 2016). HBO is also demonstrated to improve nerve regeneration of peripheral nerve injury (Oroglu et al., 2011). The beneficial effects are partly attributed to some biological activities in anti-oxidative (Li et al., 2008; Yang et al., 2010), anti-inflammatory (Lin et al., 2012; Yang et al., 2015), and anti-apoptotic (Lu et al., 2013; Wee et al., 2015) activities. Oxidative stress, inflammation and apoptosis are common features resulted from the polyQ aggregation (Bertoni et al., 2011). Therefore, we speculate that HBO therapy is highly potential to be an adjunctive therapy for the treatment of polyQ SCA diseases.

In this study, we evaluated the HBOT on the SCA17 mice and our results have shown that HBO treatment (2.2 ATA for 14 days) effectively ameliorated both the motor incoordination and cognitive impairment of SCA17 mice. We elucidated several aspects of HBO treatment on these mice, including the molecular mechanism. Furthermore, to test whether a mild conditioned HBO (M-HBO) treatment also has beneficial effect, we also conduct a 1.4 ATA HBO for 14 days on the SCA17 mice. Both of the motor coordination and memory of SCA17 mice were also improved. Our study proves the safety and activity of HBOT on SCA17 mice. It's speculated the effective treatment identified for SCA17 could also be applied to other polyQ diseases since a lot of studies have revealed the similarity in disease mechanisms and their possible treatment strategies for polyQ diseases (Bowman et al., 2005; Colomer Gould, 2005; Rego and de Almeida, 2005).

Materials and Methods

Animals and ethics statement

All of the animal experiments were conducted according to the Institutional Animal Care and Use Committee (IACUC) of the National Taiwan Normal University Taipei, Taiwan (Permit Number: NTNU107034). FVB/N mice were purchased from the National Breeding Centre for Laboratory Animals, Taipei, Taiwan. The mice were housed in individual ventilated cages (IVC) with free access to food and water in a 12-h light/dark cycle (7 a.m. to 7 p.m.). SCA17 transgenic mice with human TBP (hTBP)-109Q were maintained by breeding heterozygous male mice with FVB/N wild-type female mice as described (Chang et al., 2011; Chang et al., 2016). SCA17 mice showed ataxia around 6 weeks of age, whereas Purkinje cell degeneration

occurred at 4 weeks of age (Chang et al., 2011; Chang et al., 2016; Lin et al., 2020). Every effort was made to minimize any animal suffering.

HBOT experiments

The regular HBOT experimental timeline is shown in Figure 1A. Six-month old SCA17 transgenic mice (TG) and their wild-type littermates (WT) were divided into 4 groups (WT-control, WT-HBO, TG-control, and TG-HBO, n = 9/group). Before and after the HBOT, behavioral tests including the rotarod and Y-maze were conducted to evaluate the motor and cognitive function of mice. Mice were administered 100% oxygen at a pressure of 2.2 ATA in a chamber (HOFA Sigma105, Taipei, Taiwan) 90 min daily for 14 days. Both compression and decompression rate were 1.2 kg/cm^2. The results of the behavioral testing (III) conducted 2 months after the first round HBOT show no more improvement in motor and cognitive function of mice compared to the first and second time behavior tests. The second round of HBOT with identical condition (2.2 ATA, 90 min daily for 14 days) therefore was conducted in the mice again to evaluate the reproducible effect of HBOT. Mice were sacrificed for pathological analyses after the behavior testing (IV) proceeding the second round HBOT. To understand the toxicity of HBOT in the mice, the mouse body weight was measured every week. liver and kidney pathologies were analyzed by serum biochemistry and hematoxylin and eosin (HE) staining.

Rotarod task

Rotarod task is commonly used to evaluate the motor coordination of rodents. SCA17 transgenic mice and their wild-type littermates in this study were analyzed by the rotarod (UGO, Basile, Italy) task 2 days before and after HBOT. The mice were handled 2 min per day for 2 days before the first time rotarod task to reduce their anxiety. The analysis started with a pretraining session in which the mice were habituated on the rod for 60 sec and then trained 3 times at 4 rpm for 60 sec with a 10-min interval. Mice were tested under accelerating speed from 4 to 30 rpm for the first 5 min and 30 rpm for another 5 min. The rotarod task was conducted 3 trials per day for 2 days. The latency to fall was recorded and performance of each mouse was averaged from the six trials.

Y-maze

Y-maze is a behavioral test for measuring the willingness of rodents to explore new environments and assessed as an indicator of short-term memory. The apparatus and conduction in this study were described as previously (de Sousa et al., 2018; Huang et al., 2019b; Huang et al., 2019a). Each mouse was placed in the central space of maze

and allowed to explore freely for 8 min. The spontaneous alternation percentage was calculated by [successive entries / (total arm entries - 2) × 100] as an index of short-term memor of each mouse.

Western blot

Mouse cerebellar proteins were extracted with RIPA buffer and used in western blot analysis as previously described (Huang et al., 2015; Huang et al., 2018). Membranes were incubated with primary antibodies (Table 1) at 4°C for 12-16 hr and secondary antibodies (Table 1) for 1 hr at room temperature. The signals were developed using an ECL kit (Millipore, MA, USA). Blots were scanned by a LAS-4000 chemiluminescence detection system (Fujifilm, Tokyo, Japan). GAPDH was used as a loading control and the quantification of band density was performed using Gel-Pro Analyzer software (GelPro32; Media Cybernetics, Rockville, MD, USA) (n=3/group).

Immunofluorescent and immunohistochemistry (IHC) staining

After anesthetized with avertin (0.4 g/kg body weight), the mice were perfused with saline, and fixed with 4% paraformaldehyde (PFA). Each cerebellum was then post-fixed in 4% PFA for 4 h, followed by 10% sucrose solution for 1 h, 20% sucrose solution for 2 h, and 30% sucrose solution for 16-18 h at 4°C. The cerebellum was cut into 30-μm sections (CM3050S, Leica, Nussloch, Germany). Immunofluorescent staining was performed as described (Chen et al., 2015; Chang et al., 2016). The cerebellar sections were incubated at 4°C for 12-16 hr with primary antibodies (Table 1), followed by room temperature for 2 hr in secondary antibodies (Table 1). The cerebellar sections were mounted on gelatin-coated slides for observation under a confocal microscope (LSM 880, Zeiss). In each experiment, 4-6 sections per mouse were analyzed (n = 3/group). IHC staining was performed as previously described (Chen et al., 2015; Chang et al., 2016). The cerebellar sections were incubated in 3% H_2O_2 to suppress endogenous peroxidase and blocked with 5% normal serum, incubated with primary antibody Iba1 (Table 1), and secondary antibody (Table 1). The images were captured with a microscope (SP2, Leica). The intensity of the microglial signal was calculated by Image J (n=3-4/group).

Total antioxidant status assay

To determine whether HBOT affect the antioxidant activity in mouse, we performed a colorimetric assay with antioxidant assay kit (Cayman) to measure the total antioxidant capacity of mouse serum according to the instruction of kit. In this assay, aqueous and lipid soluble antioxidants were not separated, thus the combined antioxidant activities of all the constituents in each mouse serum are assessed.

Statistical analysis

Data were shown as mean ± SEM. An independent t-test and one-way analysis of variance (ANOVA) were performed with SPSS software to evaluate the significance. A p value cutoff of 0.05 was considered statistically significant.

Results

The effect of HBOT on mouse behavior and the treatment persistence

The results of the OF test showed that the hyperactivity of SCA17 TG mice was not improved by HBOT (Figure 1B). However, HBOT significantly reduced the anxiety of TG mice (Figure 1C). According to the results of Y-maze test, the 1st HBOT significantly attenuated the short-term memory of TG mice (Figure 1D); however, the effect only lasted for 1 month (Figure 1D). In addition, the 2nd HBOT showed no effect (Figure 1D).

From the gait analysis, we found that the step regularity of both WT and TG mice was increased right after the the 1st HBOT (Figure 1E); however, which was not lasted in the subsequent analyses performed 1-2 months later (Figure 1E). The results of the analysis of footprint position reveal that HBOT could not improve the instability of the mouse in either their right or left feet (Figure 1F-G).

The results of rotarod test indicate that both the 1st and 2nd HBOT significantly improved the motor coordination of TG (Figure 1H). The effect of 1st HBOT lasted for 1 month (Figure 1H). According to the results of behavior analysis, HBOT can improve the anxiety, spatial short-term memory ability, and motor activity of six-month-old SCA17 TG mice. The 1st HBOT can last for 1-2 months; while the second HBOT only improved short-term spatial memory and motor function.

The effect of HBOT on the expression levels of calbindin and GFAP in the cerebellum of mice

SCA17 TG mice PC reduction when they are 4 weeks old, as well as astrogliosis (Chang et al., 2011; Lin et al., 2020). West analysis was performed to detect the expression levels of calbindin (PC marker) and GFAP (astrocyte marker) in the mouse cerebellum after HBOT. The results showed that the level of calbindin in the cerebellum of TG was significantly lower than that of WT mice, while the reduction was not alleviated by HBOT (Figure 2A). HBOT significantly decreased the up-regulated GFAP expression level in TG male but not female mice (Figure 2B).

Effect of HBOT on oxidative stress in mice

HBOT may cause oxidative stress to produce more free radicals and cause cell

damage, aging and even death. Therefore, the levels of oxidative stress were detected by Western blot with SOD1 and SOD2 antibodies and oxidative stress test kit. There was no difference in SOD1 in the cerebellum among the groups (Figure 3A). The expression of SOD2 in cerebellar mitochondria was increased in male mice after HBOT, indicating that TG-HBOT male mice have a higher antioxidant capacity. However, the increased SOD2 level in female TG mice was reduced by HBOT (Figure 3B). Assay with the oxidative stress detection kit show that the levels of antioxidants in the mouse serum were similar among the groups, indicating that HBOT did not cause the oxidative stress (Figure 3C).

The effect of HBOT on neuronal survival and cognitive-related proteins

Many reports have pointed out that HBOT promotes neurogenesis, cell proliferation and cognition. The activation of cerebellar pERK (Thr202/Tyr204) in TG mice was further promoted by HBOT (Figure 4A). The highly down-regulated pCaMKII (Thr286) in TG male mice was significantly increased by HBOT (Figure 4B). In addition, HBOT also activated pAKT (Ser473) in male TG mice (Figure 4C). All of the activation of pERK, pCaMKII and pAKT may further activate pCREB (Ser133). We found that pCREB in both TG male and female mice was significantly activated (Figure 4D). pCREB (Ser133) activation may induce the expression of BDNF (proBDNF) and NGF (proNGF); we found that BDNF but not NGF was up-regulated in TG mice by HBOT (Figure 4E-F); in addition, the expression levels of proNGF and mNGF by HBOT did not differ between male and female mice (Figure 4F). It's pointed out that pAKT (Ser473) may directly promote the increase of $GABA_A$ receptors (Wang et al., 2003; Trujeque-Ramos et al., 2018). $GABA_A$ receptors are increased in TG female mice by HBOT (Figure 4G).

The effect of HBOT on the chaperone-mediated autophagy (CMA) protein HSC70

HBOT significantly increased the HSC70 in TG male mice; while TG female mice had much lower HSC70 than WT female mice, which was not attenuated by HBOT (Figure 5).

The effect of HBOT on the expression of dendritic growth-related protein GAD67

HBOT was reported to promote the growth of dendrites of nerve cells. The GAD67 was detect significantly increased in the TG male mice by HBOT, but no difference was found between female mice (Figure 6).

The effect of HBOT on mouse cerebellar protein expression by immunofluorescence staining

The results of immunofluorescence staining show that the HBOT increased the nerve fiber density, lobe width, and PC numbers and cell bodies in TG mice (Figure 7A-C). The activation of pERK (Thr202/Tyr204) in lobe are scattered in the PC, molecular and granular layers; quantitative results show WT-HBOT is significantly lower than WT-Ctrl male mice, TG-Ctrl and TG-HBOT male mice are significantly higher than WT-Ctrl male mice; while TG-HBO female mice are significantly higher than WT-Ctrl female mice (Figure 7A-B, D).

The s100 protein, which helps cell proliferation, shows stronger signals in TG than in WT mice, and the signal is scattered in the PC and granular layers. Quantitative results show that s100 in TG-HBO male and TG female mice increased significantly (Figure 7A-B, E).

The pAKT (Thr308) signal is shown in the molecular layer of WT mice while it is in the molecular and granular layers of TG mice. However, HBOT shows no effect between the respective groups of WT and TG (Figures 7F-I).

TG mice has a higher level of astrogliosis than TG mice as previous reported. According to the statistical results of fluorescence intensity, TG-HBO female mice were significantly lower than TG-Ctrl female mice, indicating that HBOT can ameliorated the inflammation of astrocyte activation (Figure 7F-G, J).

pCaMKII (Thr286) signal appears on the PC and the granular layers. TG female mice are significantly lower than WT-Ctrl female mice. HBOT has no significant effect on the signal (Figure 7K, L, O).

The pCREB (ser133) signal appears in the molecular and granular layers. The quantitative results of fluorescence intensity show that TG-HBOT male mice have a significant increase the signal compared with TG-Ctrl male mice, while WT-HBO female mice are higher than WT-Ctrl female mice (Figure 7M-N, P).

The 1TBP18 signal is concentrated in the PC nucleus. The quantitative results of fluorescence intensity show that HBOT has no effect on thesignal levels (Figure 7K, N, Q).

Evaluation of the effect of HBOT on microglia of mouse cerebellum

According to the results of IHC staining, TG mice have a significant higher microglia activation compared with WT mice. HBOT significantly decrease the signal in TG male but not female mice (Figure 8).

Discussion

SCA17 TG mice was established using transgene TBP-109Q driven by pcp2 promoter, which causes the abnormal expansion polyQ protein accumulated in the cerebellar PC, and further leads to neurodegeneration. The sympotoms include ataxia caused by cerebellar atrophy, stiffness and atrophy, nerve cell dendritic deficit, neuronal death, apoptosis and cognitive dysfunction and inflammation caused by the proliferation of glial cells (Chang et al., 2011; Chang et al., 2016; Lin et al., 2020). HBOT can promote angiogenesis (Heng et al., 2000) (Heng et al ., 2000; Hadanny et al., 2018), promote neurogenesis (Lin et al., 2012; Ince et al., 2016), inhibit inflammation and inhibit cell apoptosis (Shams et al., 2017), improve cognition dysfunction (Chen et al., 2016), and functions in promoting skeletal muscle growth (Nagatomo et al., 2018). Therefore, we would like to evaluate the therapeutic potential of HBOT on SCA17.

In this study, we explored the effect of 2.2 ATA HBOT on 6-7 month- old SCA17 TG mice. Through behavioral tests, including OF, Y-maze, gait analysis, and rotarod, we found that the HBOT could not reduce the hyperactivity but reduced the anxiety of TG-HBO mice. HBOT also increased the alternative translocation rate of mice in the Y maze, which indicates that HBOT improved short-term memory of TG mice. It's reported from a HBOT clinical trials, 154 cases with severe traumatic brain, their cognitive function were significantly improved after HBOT (Hadanny et al., 2018). The results of gait analysis show HBOT only improved the regularity of gait. However, it's interesting to find HBOT significantly increased the latency of TG mice in the roterod test, which is the most important index for ataxia improvement. This data indicates that HBOT might improve the grasping ability and muscle endurance of SCA17 mice. It is known that HBOT can regenerate muscle fiber cells in mice with muscle injury caused by ischemia (Asano et al., 2007). Another experiment also identified HBOT reduced the muscle necrosis and edema of the ischemia hind limbs of rats (Nylander et al., 1985). HBOT also reduced the degree of muscle pain and the interference during exercise of 41 athletes with muscle injury (Chen et al., 2019b). These reports indicate that HBOT helps to recover the muscle strength.

The HBOT persistence analysis, first, the results of the OF analysis showed that TG mice reducing anxiety after HBOT for more than 1 month; second, the performance in Y-maze in TG-Ctrl mice can lasted for more than 1 month; third, in the roller test, the HBOT effect on TG mice lasted until the third month test from these result, we speculate that HBOT can lasted for 1-2 months. However, there is no literature suggest the persistence of a single course of HBOT. The second course of HBOT treatment only improved the short-term memory of mice. It may be because the mice were 10 months old and the decline condition of motor and muscle strength was hard to be recovered.

Many literatures have shown that HBOT may cause excessive ROS, which may lead to apoptosis and aging (Dennog et al., 1996; Benedetti et al., 2004; Korpinar and Uzun, 2019). Our results show the SOD2 protein is reduced in female TG-HBO mice. The analysis using the oxidative stress kit shows that there is no oxidative stress in the mice. The result indicates that the HBOT dose does not cause excessive ROS in mice.

The analysis of the expression level of PC protein calbindin in the cerebellum of SCA17 TG mice, we found that HBOT could not effectively prevent the loss of PC. However, through the results of immunofluorescence staining of cerebellar sections, we found that the nerve fibers of PC in TG-HBO mice were relatively denser as well as the fluorescent intensity of IP3R1 has a significant improvement. Evaluation of neuroinflammation with immunostaining of gliosis markers GFAP and Iba1, we found GFAP and Iba1 expressions were only reduced in male TG mice by HBOT.

To study the molecular mechanism of the therapeutic effect of HBOT, we analyzed the activation of pAKT, pERK, pCaMKII, pCREB, BDNF and NGF, which are molecules commonly involved in the cell proliferation and neuroprotective pathways. Based on these results, we proposed a possible HBOT Diagram of the molecular mechanism of action (Figure 9). It was reported that HBOT affects the activation of pAKT (Ser473/Thr308) (Lin et al., 2014), which in turn increases the expression of $GABA_A$ receptors (Wang et al., 2003). GABA is the main inhibitory neurotransmitter, which plays a vital role in regulating the balance of excitatory inhibition in the mammalian brain. GABA has three types of receptors, ionic $GABA_A$ and $GABA_C$, and metabolic $GABA_B$ (Chebib and Johnston, 1999). Both $GABA_A$ and $GABA_C$ receptors have shown to have neuroprotective effects in the experimental mode of middle cerebral artery occlusion (MCAO) (Liu et al., 2015); through activation of pAKT (Ser473/Thr308), pERK (Thr202/Tyr204) and pCREB (Ser133), the expression levels of mBDNF and mNGF are up-regulated, which help neuron growth and differentiation (Wang et al., 2018).

It is known that HBOT can ameliorate the cognitive impairment and hippocampal atrophy of AD mice, and can also increase the activation of pCREB and the expression of BDNF promoter (Choi et al., 2019); in addition, BDNF can also trigger TrkB-mediated Phosphorylation of $GABA_A$ receptor on the surface of PC (Cheng et al., 2005; Huang et al., 2012); in our experiments, we found that pAKT, pERK and PCREB activation and BDNF and $GABA_A$ receptors levels were all increase in SCA17 mice; also increase.

pAKT promotes cell survival and cell proliferation. In the fluorescence staining of cerebellar sections, we found that pAKT (Thr308) of TG mice is expressed in the PC layer, which is different from other groups, which may be a compensation mechanism for PC loss due to the accumulation of polyQ in PC. According to the

results of our previous study, gliosis in SCA17 TG mice could be due to the activation of pERK, which makes astrocytes and microglia proliferate and leads to the path of neuronal apoptosis (Lin et al., 2020). We found astrocytes of TG mice have decreased, we speculate that the activation of pERK may not involve in gliosis. It's reported that HBOT can promote the increase of vascular endothelial growth factor (VEGF) through pERK activation (Lee et al., 2006), so the activation of pERK in TG-HBO mice may also lead to vascular proliferation.

Through western blot, we found that the activation of pCaMKII (Thr286) in TG-HBO mice was increased, which might be that HBOT increased the synaptic Ca^{2+}, promoted the activation of CaMKII and in turn increased the activation of pCREB and the expression of BDNF, finally achieved the effect of nerve repair as other studies (Dan et al., 1999; Ma et al., 2014).

Our previous study found that GAD67 in the cerebellum of SCA17 mice was decreased (Chang et al., 2016), and GAD67 was also reduced in HD cell culture (Guo et al., 2013). The expression level of GAD67 in SCA17 TG male mice was increased after HBOT. It is known that synthesis of GABA is regulated by GAD65 and GAD67, and the function of GABA is to maintain the balance of CNS excitation and inhibition (Li et al., 2008).

We also found that the s100 fluorescence intensity of TG-HBO mice was increased. S100 is an inhibitor of apoptosis and a stimulator of cell proliferation in the process of neurodegeneration (Donato et al., 2009). The increase of s100 in SCA17 TG mice can be regarded as a compensation mechanism for mice response to apoptosis. S100 treatment of sciatic nerve transection can reduce neuron death (Iwasaki et al., 1997). HBOT treatment also increased s100 in sciatic nerve transection experiments (Shams et al., 2017).

In neurodegenerative diseases AD and PD, the activation of CMA can be observed to be a neuroprotective effect (Shacka et al., 2008). The activation of CMA can protect nerve cells from death by removing abnormally folded proteins. Both HSC70 and LAMP-2A proteins will be increase during CMA (Periyasamy-Thandavan et al., 2009). We have found that the increase of chaperone protein and autophagy can enhance the clearance of mutant protein aggregation and slow down SCA17 pathological progress of mice (Chang et al., 2016). Expression of HSC70 in TG-HBO male mice was significantly increase, which reveals that HBOT may promote the male mouse autophagy, and reduced the mutant polyQ protein accumulation.

Summary from protein quantitative and histopathological staining shows that HBOT can promote CMA, reduce PC death, and inhibit nerve inflammation in SCA17 TG mice, which may improve cognition. In addition, oxidative stress test, complete blood count test, blood biochemistry and the results of liver, and kidney

pathological examination indicated that HBOT is a safe treatment. Therefore, we provide a therapeutic option for polyQ diseases that currently have no effective treatment.

Figure legends

Fig. 1 The effect of HBOT on mouse behavior and treatment persistence. (A) Experimental timeline for analyzing HBOT effect persistence. (B) Total moving distance of different groups. (C) Center dwell time of different groups. (D) Y-maze analysis result of different groups. (E) Footstep regularity of different groups. (F) Right paw gait gap of different groups. (G) Left foot gait gap of different groups. (H) Rota-rod test analysis results of different groups. (*: compared to WT ctrl, #: compared to TG ctrl；*P < 0.05, **P<0.01, ***P < 0.001，Male N=4；Female N=5).

Fig. 2 To analyze the effect of HBOT on the expression of calbindin and GFAP protein in cerebellum of SCA17 TG mice. (A) Calbindin protein expression. (B) GFAP protein expression. (*: compared to WT ctrl, #: compared to TG ctrl；*P < 0.05, **P<0.01, ***P < 0.001, $^{\#}$P < 0.05，Male N=3；Female N=3)

Fig. 3 Analysis of the effect of HBOT on oxidative stress in mice. (A) Analysis of cytoplasmic antioxidant enzymes in mice cerebellum with SOD1 antibody (B) Analysis of anti-oxidant enzymes in granules of mice cerebellum with SOD2 antibody (C) Detection of antioxidant activity in mice by oxidative pressure kit The degree of stress. (*: compared to WT ctrl, #: compared to TG ctrl；*P < 0.05, **P<0.01, $^{\#}$P < 0.05，Male N=3；Female N=3)

Fig. 4 To analyze the effect of HBOT on promoting mice cerebellar nerve cell proliferation and improving the performance of cognitive impairment related proteins. (A) pERK (Thr202/Tyr204)/ERK ratio (B) pCaMKII (Thr286)/CaMKII ratio (C) pAKT (ser473)/AKT ratio (D) pCREB (ser133)/CREB ratio (E) The ratio of pro-BDNF/GAPDH and m-BDNF/GAPDH protein expression. (F) the ratio of pro-NGF/GAPDH and m-NGF/GAPDH protein expression (G) the amount of protein expression of $GABA_A$ receptor after HBOT. (*: compared to WT ctrl, #: compared to TG ctrl；*P < 0.05, **P<0.01, ***P < 0.001, $^{\#}$P < 0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P < 0.001，Male N=3；Female N=3)

Fig. 5 To analyze the effect of HBOT on the performance of Chaperone-mediated autophagy (CMA) related protein HSC70 .

The amount of HSC70 in the cerebellum of the mice was analyzed in the CMA pathway. (*: compared to WT ctrl, #: compared to TG ctrl；*P < 0.05, **P<0.01, ##P<0.01，Male N=3；Female N=3)

Fig. 6 To analyze the effect of HBOT on the expression of dendritic growth-related protein GAD67 in cerebellum of mice. GAD67 protein expression. (*: compared to WT ctrl；**P<0.01，Male N=3；Female N=3)

Fig. 7 Evaluation of the effect of HBOT on cerebellar protein expression in mice by immunofluorescence staining of cerebellum tissue sections. (A) The expression of IP3R, pERK Thr202/Tyr204 and s100 in the cerebellum of male mice (B) The expression of IP3R, pERK Thr202/Tyr204 and s100 in the cerebellum of female mice (C) Fluorescence intensity of IP3R1 (D) Fluorescence intensity of pERK (Thr204/Tyr202) (E) Fluorescence intensity of s100 (F) The expression of IP3R, pAKT (Thr308) and GFAP in the cerebellum of male mice (G) The expression of IP3R, pAKT (Thr308) and GFAP in the cerebellum of female mice (20×) (H) The expression of IP3R, pAKT (Thr308) and GFAP in the cerebellum of female mice (40×) (I) Fluorescence intensity of pAKT (Thr308) (J) Fluorescence intensity of GFAP (K) The expression of IP3R, pAKT (Thr286) and 1TBP18 in the cerebellum of male mice (L) The expression of IP3R, pCaMKII (Thr286) and GFAP in the cerebellum of female mice (M) The expression of IP3R, pCREB (ser133) and s100 in the cerebellum of male mice (N) The expression of IP3R, pCREB (ser133) and 1TBP18 in the cerebellum of female mice (O) Fluorescence intensity of pCaMKII (Thr286) (P) Fluorescence intensity of pCREB (ser133) (Q) Fluorescence intensity of 1TBP18. (*: compared to WT ctrl, #: compared to TG ctrl；*P < 0.05, **P<0.01, ***P < 0.001, $^{\#}$P < 0.05，Male N=3；Female N=3)

Fig. 8 Cerebellar tissue sections for IHC staining to evaluate the effect of HBOT on mice cerebellum microglia and quantitative results. (*: compared to WT ctrl, #: compared to TG ctrl；*P < 0.05, **P<0.01, #P < 0.05，Male N=3；Female N=3)

Fig. 9 The hypothesis of molecular mechanism of HBOT in SCA17 mice of this study.

108年度專題研究計畫成果彙整表

計畫主持人：謝秀梅	**計畫編號：**108-2320-B-003-005-
計畫名稱：高壓氧治療SCA17小鼠之持久性、再現性、強度與分子效應	

成果項目			量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文	0	篇	
		研討會論文	0		
		專書	0	本	
		專書論文	0	章	
		技術報告	0	篇	
		其他	0	篇	
國外	學術性論文	期刊論文	0	篇	
		研討會論文	0		
		專書	0	本	
		專書論文	0	章	
		技術報告	0	篇	
		其他	0	篇	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	2		江孟格、莊欣穎
		博士生	0		
		博士級研究人員	0		
		專任人員	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士級研究人員	0		
		專任人員	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					