

Hyperbaric Oxygen and Ginkgo Biloba Extract Ameliorate Cognitive and Memory Impairment via Nuclear Factor Kappa-B Pathway in Rat Model of Alzheimer's Disease

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Abstract

Background: Hyperbaric oxygen (HBO) and Ginkgo biloba extract (e.g., EGB 761) were shown to ameliorate cognitive and memory impairment in Alzheimer's disease (AD). However, the exact mechanism remains elusive. The aim of the present study was to investigate the possible mechanisms of HBO and EGB 761 via the function of nuclear factor kappa-B (NF- κ B) pathway.

Methods: AD rats were induced by injecting β -amyloid 25–35 into the hippocampus. All animals were divided into six groups: Normal, sham, AD model, HBO (2 atmosphere absolute; 60 min/d), EGB 761 (20 mg·kg⁻¹·d⁻¹), and HBO/EGB 761 groups. Morris water maze tests were used to assess cognitive, and memory capacities of rats; TdT-mediated dUTP Nick-End Labeling staining and Western blotting were used to analyze apoptosis and NF- κ B pathway-related proteins in hippocampus tissues.

Results: Morris water maze tests revealed that EGB 761 and HBO significantly improved the cognitive and memory ability of AD rats. In addition, the protective effect of combinational therapy (HBO/EGB 761) was superior to either HBO or EGB 761 alone. In line, reduced apoptosis with NF- κ B pathway activation was observed in hippocampus neurons treated by HBO and EGB 761.

Conclusions: Our results suggested that HBO and EGB 761 improve cognitive and memory capacity in a rat model of AD. The protective effects are associated with the reduced apoptosis with NF- κ B pathway activation in hippocampus neurons.

Key words: Ginkgo Biloba Extract 761; Hyperbaric Oxygen; Nuclear Factor Kappa-B; Rats; Alzheimer's Disease

INTRODUCTION

Alzheimer's disease (AD) is a devastating neurodegenerative disease characterized by progressive cognitive impairment and psychobehavioral disturbances or language impairment. Accumulating evidence indicated that excess production of β -amyloid (A β) occurs early in the disease progression, it may represent a crucial step in AD pathogenesis.^[1] Recently, it has been demonstrated that there is a close link between nuclear factor kappa-B (NF- κ B) and nerve degeneration diseases such as AD, Parkinson's disease (PD), and Huntington's disease (HD).^[2] In the early stage, the activation of NF- κ B pathway with deposition of amyloid was observed in neurons and astrocytes in human ADs.^[3] However, the exact function of NF- κ B activation in such context remains unclear.

The concentrated extracts of Ginkgo biloba leaves (EGB 761) have been found to protect against various neural and vascular damages.^[4,5] EGB 761 is a well-known anti-oxidant which inhibits A β -derived fibril formation and apoptosis. In addition, the herb can modulate brain cholinergic transmission, increase brain cholinergic activity, and normalize the acetylcholine receptors in the hippocampus area.^[6] Hyperbaric oxygen (HBO)

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therapy ameliorates the disease course of a number of human diseases such as hypoxia, ischemia, and reperfusion. Further, recent studies have shown that the HBO therapy reduces spinal cord injury (SCI) and facilitates recovery of neurological functions.^[7,8] In this regard, it has been reported that HBO exerts its protective effect through TLR2/NF- κ B pathway inhibition and consequent suppression of pro-inflammatory cytokine release.^[9] In this study, we analyzed the biological effects of A β 25–35 on neurons, which is an active fragment of toxic A β (A β 1–40 and A β 1–42).^[10] Further, we investigated the potential role of NF- κ B in mediating protective effects of HBO/EGB 761 against cognitive and memory impairment using a rat model of AD.

METHODS

Animals

Seventy-two male Sprague-Dawley rats (age: 5–6 months and weight: 250–350 g) were obtained from Shanghai Laboratory Animal Center. The animals were acclimatized for 7 days at 23°C with a 12-h light-dark cycle and allowed free access to drinking water and pellet diet. All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the Southeast University (Nanjing, Jiangsu, China), and all efforts were made to minimize the number of used animals as well as their suffering.

Preparation of β -amyloid 25–35 and Ginkgo biloba extract 761

A β 25–35 (A4559, Sigma-Aldrich, USA) was resolved in sterile saline solution at 37°C for 7 days for the preparation of aggregated A β 25–35 (1 g/L). EGB 761 (40 mg/tablet, German pharmaceutical company, Dr. Weimashupe, Germany) was prepared in drinking water (20 mg/ml).

Injection of aggregated β -amyloid 25–35

In brief, all animals were anesthetized by intraperitoneal injection of chloral hydrate (300 mg/kg; Tianjin Chemical Reagent Development Center, China). Surgery and injection of A β 25–35 was carried out as previously described.^[11] The aggregated A β 25–35 (10 μ l; 1 μ l/min) was injected into bilateral hippocampus. The sham control was injected with the same amount of saline. Postoperatively, animals were treated with prophylactic antibiotics (penicillin; 80,000 unit/0.25 ml) for 3 days. After 3 weeks, animals were randomly assigned into six groups (n = 12/each group): Normal, sham, AD model, HBO, EGB 761, and HBO/EGB 761 groups according to different treatment regiments (see below).

Hyperbaric oxygen and Ginkgo biloba extract 761 administrations

The gavage of EGB 761 was performed in the EGB 761 group as previously described.^[12] For HBO treatment in the HBO group, 100% oxygen was administered at a pressure of 2.0 atmosphere absolute in a hyperbaric chamber (SHC3200–8500, Shanghai 701 Yang garden hyperbaric oxygen chamber Co Ltd., China) for 1 h including 15 min of compression and decompression. Depending on

experimental design, EGB 761 and HBO was administrated in the HBO/EGB 761 group. The treatment groups (HBO, EGB 761 and HBO/EGB 761) consisted of two courses (10 days per course) with an interval of 3 days between two courses. There was no treatment with the other groups (normal, sham, AD model). Moreover, all of the animals were bred with food pellets and water throughout the experiment.

Morris water maze

The Morris water maze was performed in a circular water tank (160 cm \times 50 cm) filled with water to a depth of 25 cm. The tank was divided into four quadrants (I, II, III, and IV). A platform was placed inside the quadrant I. The hidden platform (from day 1 to day 6) and probe trial (on day 7) was performed. For the hidden platform trial, rats were tested twice per day. Specifically, rats were placed in the water at the starting location (quadrants II and III). The trial was considered to be successful when they managed to escape to the platform within 90 s. If a rat failed to escape within 90 s, the escape latency was recorded as 90 s. All rats were allowed to spend 20 s on the platform and returned to their cages. The escape latency, swimming speed, and trajectory were recorded for each rat. For the probe trial, the platform was removed and the starting point was located in quadrant III. Within 90 s, the number of crossings time spent in the quadrant I were record.

TdT-mediated dUTP Nick-End Labeling staining

After trials, rats (n = 6/each group) were immediately anesthetized and perfused with 0.01 mol/L phosphate buffer (pH 7.4), followed by ice-cold 4% paraformaldehyde through the left ventricle. Brains were removed and embedded in paraffin. Sections were taken from each brain for TdT-mediated dUTP Nick-End Labeling (TUNEL) test. The process was conducted according to the manufacturer's instructions of Tunel kit (Nanjing KeyGEN Biotech. Co., Ltd., Jiangsu, China). The data were represented as the apoptotic index (AI = apoptosis/total cellular score \times 100%) in the CA1 of hippocampus.

Western blotting analysis

The protein of hippocampus tissue was prepared using tissue isolation kit and measured using BCA kit (Beyotime Biotechnology, Jiangsu, China). Samples of protein (15 mg) were subjected to dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis and were transferred onto polyvinylidene fluoride membranes. Membranes were blocked and incubated with a set of primary antibodies (anti-p-IKK α , anti-IKK α / β , anti-IkB α , and anti-p-IkB α , Bioss Inc., USA) overnight at 4°C. The blots were visualized using an ECL detection kit (GE Healthcare, Germany). A densitometric analysis was performed.

Statistical analysis

Data were shown as mean \pm standard deviation (SD). Statistical analysis was performed using one-way or repeated measures analysis of variance (ANOVA), followed by Turkey's *post-hoc* analysis for multiple comparisons and *t*-test for dependent samples using SPSS 11.5 (SPSS Inc., Chicago, IL, USA). A value of P < 0.05 was considered statistically significant.

RESULTS

Hyperbaric oxygen/Ginkgo biloba extract 761 improves cognitive and memory capacities in rat model of Alzheimer's disease

Hidden platform trail

In the hidden platform trial, rats swam along pool wall in the 1st day. However, on the 4th day, the searching behavior performed the cognitive task. In the 6th day, rats almost had a clear goal, their trajectories were nearly single-type [Figure 1a]. The escape latency of HBO/EGb 761 group decreased significantly comparing with AD model group in the 5th day [HBO/EGb 761

group: 22.70 ± 3.08 s vs. AD model: 44.55 ± 3.74 s; $F = 25.968$, $P < 0.05$; Figure 1b]. Moreover, in the 6th day, the escape latency decreased significantly in the treatment group (HBO, EGb 761, and HBO/EGb 761 groups) compared to AD model group (HBO: 23.88 ± 3.08 s vs. AD model: 40.55 ± 3.74 s, $F = 16.671$, $P < 0.05$; EGb 761: 24.44 ± 2.78 s vs. AD model: 40.55 ± 3.74 s, $F = 18.428$, $P < 0.05$; HBO/EGb 761: 19.70 ± 3.08 s vs. AD model: 40.55 ± 3.74 s, $F = 69.692$, $P < 0.01$). Moreover, HBO/EGb 761 group had superior effects. No significant difference was observed in the swim speed in the six continuous Morris water maze test [$F = 0.886$, $P > 0.05$; Figure 1c].

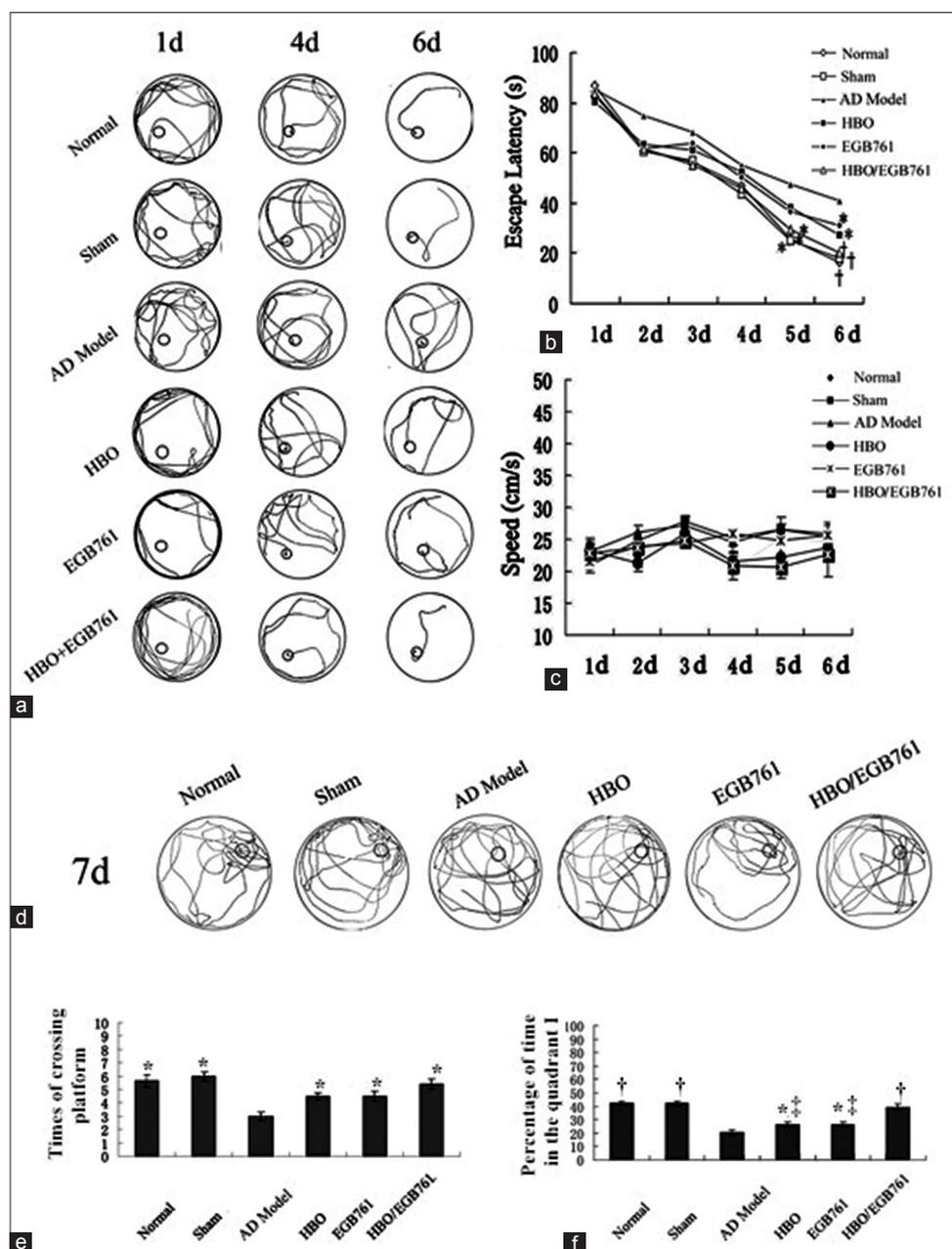


Figure 1: Hyperbaric oxygen (HBO)/Ginkgo biloba extract 761 (EGb 761) improved cognitive and memory capacities of Alzheimer's disease rats. (a) The swimming trajectories of each group in the 6 consecutive training days. (b) The escape latency of each group in the 6 consecutive training days. (c) The swimming speed of each group in 6 consecutive training days. (d) The swimming trajectories of each group in the 7th day. (e) The times of crossing the former platform position of each group. (f) Percentage of time spent in the quadrant I. * $P < 0.05$, † $P < 0.01$, versus AD model group; ‡ $P < 0.05$, versus HBO/EGb 761 group.

Probe trail

In the 7th day, rats' swimming trajectory was depicted in Figure 1d–1f. The trajectory was around the position of quadrant I. Compared with AD model group, HBO, EGB 761, and HBO/EGB 761 groups had longer time in quadrant I to cross the platform [HBO: 4.20 ± 0.29 s vs. AD model: 3.00 ± 0.30 s, $F = 8.308$, $P < 0.05$; EGB 761: 4.60 ± 0.48 s vs. AD model: 3.00 ± 0.30 s, $F = 8.113$, $P < 0.05$; HBO/EGB 761: 4.30 ± 0.37 s vs. AD model: 3.00 ± 0.30 s, $F = 8.308$, $P < 0.05$; Figure 1e]. The percentage of time in quadrant I increased significantly in HBO, EGB 761, and HBO/EGB 761 groups comparing with AD model group [HBO: $26.50 \pm 1.60\%$ vs. AD model: $20.33 \pm 1.78\%$, $F = 6.621$, $P < 0.05$; EGB 761: $26.50 \pm 1.70\%$ vs. AD model: $20.33 \pm 1.78\%$, $F = 6.218$, $P < 0.05$; HBO/EGB 761: $41.70 \pm 1.94\%$ vs. AD model: $20.33 \pm 1.78\%$, $F = 64.26$, $P < 0.01$; Figure 1f]. In this regard, HBO and EGB 761 had synergistic effects (HBO/EGB 761: $41.70 \pm 1.94\%$ vs. EGB 761: $26.50 \pm 1.70\%$, $F = 36.18$, $P < 0.05$; HBO/EGB 761: $41.70 \pm 1.94\%$ vs. HBO: $26.50 \pm 1.60\%$, $F = 34.392$, $P < 0.05$).

Hyperbaric oxygen/Ginkgo biloba extract 761 reduces apoptosis

In TUNEL staining [Figure 2a–2f], no positive staining was observed in normal and sham groups, however, a significant amount of positively stained cells was observed in AD model group. In HBO, EGB 761, and HBO/EGB 761 groups, normal

hippocampus neurons were surrounded by sporadically positive cells. The AI calculation uncovered a significant difference among these groups [HBO: $5.35 \pm 0.25\%$ vs. AD model: $23.50 \pm 1.41\%$, $F = 160.110$, $P < 0.05$; EGB 761: $5.19 \pm 0.31\%$ vs. AD model: $23.50 \pm 1.41\%$, $F = 160.960$, $P < 0.05$; HBO/EGB 761: $4.29 \pm 0.44\%$ vs. AD model: $20.33 \pm 1.78\%$, $F = 64.260$, $P < 0.05$; Figure 2g].

Hyperbaric oxygen/Ginkgo biloba extract 761 activates nuclear factor kappa-B pathway

In addition, we went on to investigate whether NF- κ B pathway is activated in our models [Figure 3]. This analysis revealed that NF- κ B pathway-related proteins such as phospho-IKK α , -I κ B α , and NF- κ B were slightly induced in model group. However, this difference did not reach statistical significance comparing with the normal, sham group (p-IKK α : Normal: $13.40 \pm 1.43\%$ vs. AD model: $11.36 \pm 0.35\%$, $F = 0.081$, $P > 0.05$; sham: $11.78 \pm 0.37\%$ vs. AD model: $11.36 \pm 0.35\%$, $F = 4.050$, $P > 0.05$. p-I κ B α : normal: $10.36 \pm 0.34\%$ vs. AD model: $11.36 \pm 0.81\%$, $F = 0.820$, $P > 0.05$; sham: $10.92 \pm 0.84\%$ vs. AD model: $11.36 \pm 0.81\%$, $F = 0.640$, $P > 0.05$. NF- κ B: normal: $10.64 \pm 0.34\%$ vs. AD model: $11.34 \pm 0.25\%$, $F = 2.690$, $P > 0.05$; sham: $10.24 \pm 0.61\%$ vs. AD model: $11.34 \pm 0.25\%$, $F = 0.645$, $P > 0.05$).

Interestingly, HBO and EGB 761 significantly activated NF- κ B pathway in hippocampus neurons. As shown in

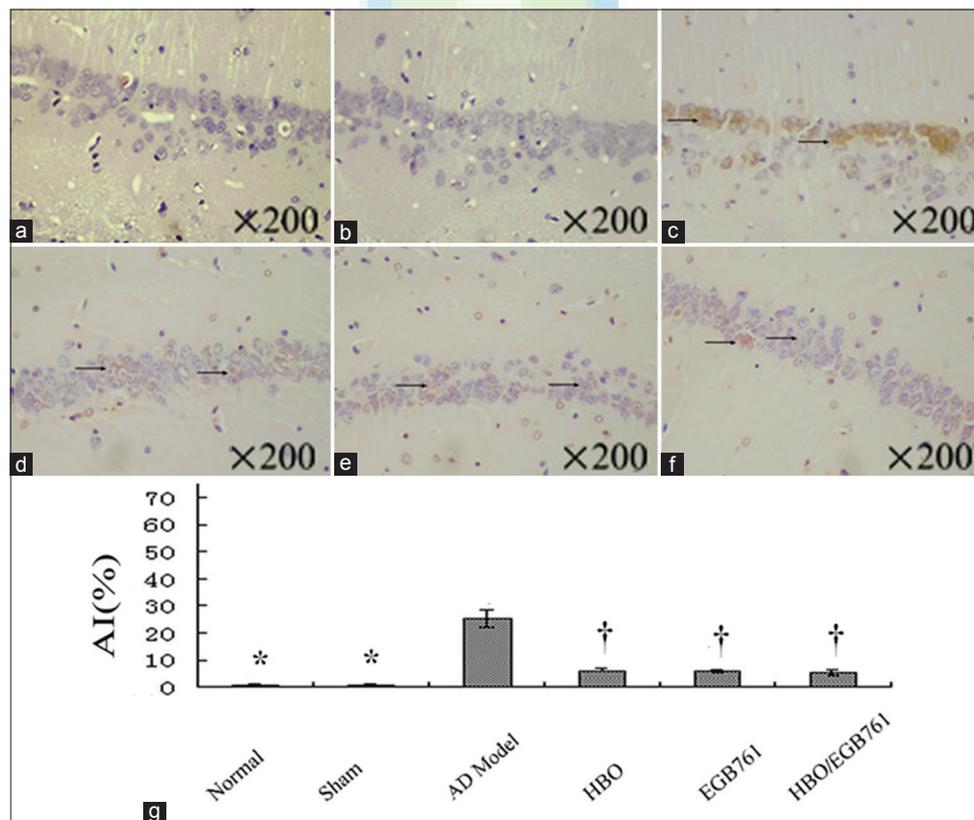


Figure 2: Hyperbaric oxygen (HBO)/Ginkgo biloba extract 761 (EGB 761) reduces apoptosis (a–f: TUNEL staining). (a and b) There was no brown precipitate (apoptosis identification) in normal and sham groups. (c) The large visible brown precipitate (arrows) was detected in the AD model group. (d–f) The normal hippocampal neurons were surrounded by the sporadic visible brown precipitate (arrows) in the HBO; EGB 761 and HBO/EGB 761 groups. (g) The apoptotic index showed the significant difference among different groups: AD Model, HBO, EGB 761 and HBO/EGB 761 groups. * $P < 0.01$, † $P < 0.05$, versus AD model group. AI: Apoptotic index.

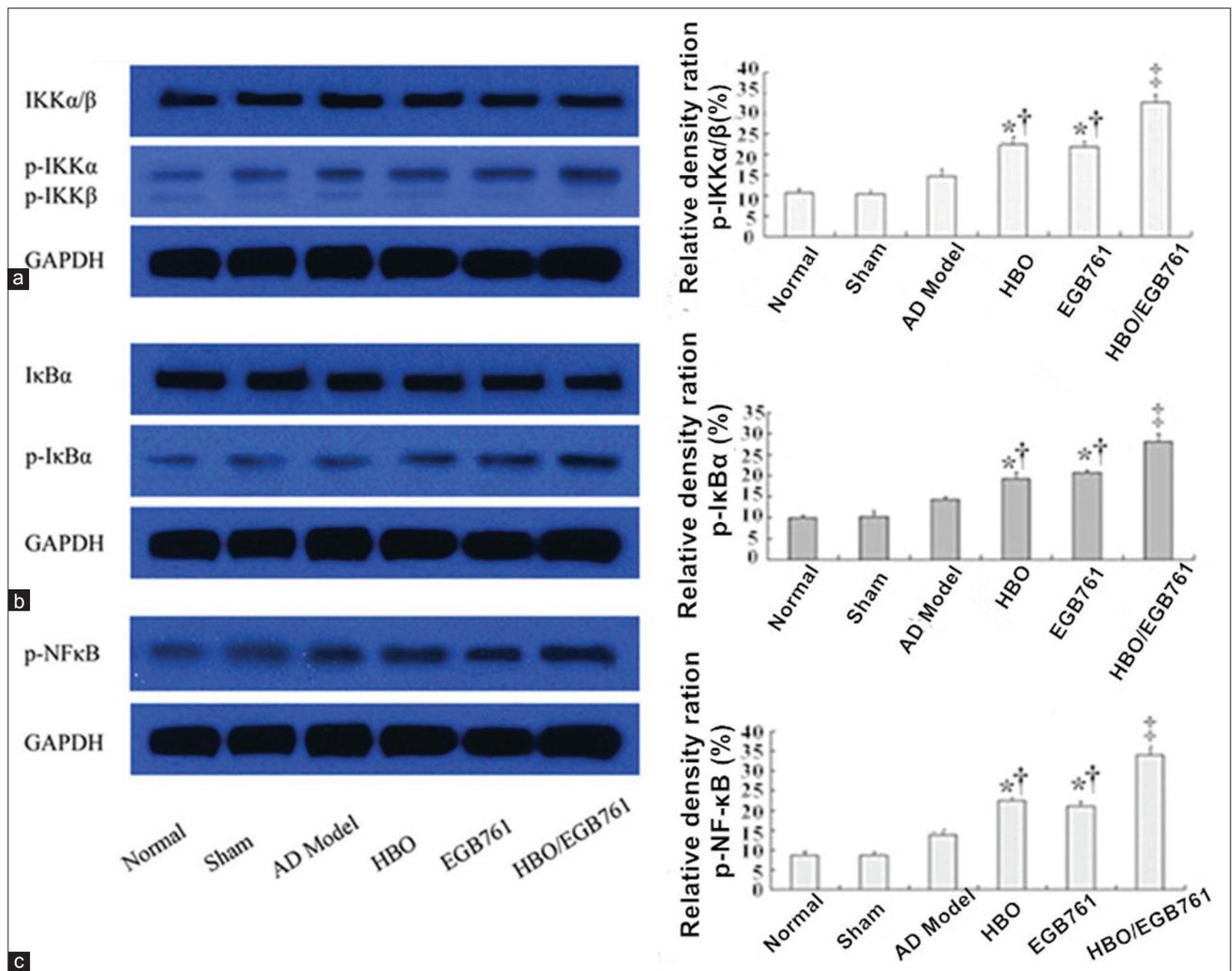


Figure 3: Hyperbaric oxygen (HBO)/Ginkgo biloba extract 761 (EGB 761) activates nuclear factor kappa-B pathway. HBO and EGB 761 significantly increased phosphorylation of IKKα/β (a), IκBα (b), and nuclear factor kappa-B (c). * $P < 0.05$, † $P < 0.01$, versus AD model group; ‡ $P < 0.05$, versus HBO/EGB 761 group.

Figure 3, comparing with the normal, sham and model groups, the expression of NF-κB-related proteins were significantly increased in HBO, EGB 761, and HBO/EGB 761 groups (p-IKKα: HBO: $15.92 \pm 0.52\%$ vs. AD model: $11.36 \pm 0.35\%$, $F = 52.780$, $P < 0.05$; EGB 761: $16.02 \pm 0.35\%$ vs. AD model: $11.36 \pm 0.35\%$, $F = 86.230$, $P < 0.05$; HBO/EGB 761: $23.92 \pm 0.38\%$ vs. AD model: $11.36 \pm 0.35\%$, $F = 576.300$, $P < 0.01$. p-IκBα: HBO: $14.18 \pm 0.09\%$ vs. AD model: $11.36 \pm 0.35\%$, $F = 60.810$, $P < 0.05$; EGB 761: $14.28 \pm 0.14\%$ vs. AD model: $11.36 \pm 0.35\%$, $F = 56.164$, $P < 0.05$; HBO/EGB 761: $24.41 \pm 0.56\%$ vs. AD model: $11.36 \pm 0.35\%$, $F = 126.310$, $P < 0.01$. NF-κB: HBO: $15.00 \pm 0.19\%$ vs. AD model: $11.34 \pm 0.25\%$, $F = 105.100$, $P < 0.05$; EGB 761: $14.64 \pm 0.59\%$ vs. AD model: $11.34 \pm 0.25\%$, $F = 53.510$, $P < 0.05$; HBO/EGB 761: $25.01 \pm 0.38\%$ vs. AD model: $11.34 \pm 0.25\%$, $F = 709.690$, $P < 0.01$).

DISCUSSION

Aging, as a risk factor for AD, has been widely investigated. The aging of the nervous system is a physiological process

characterized by chronic apoptosis of functional neurons. It is widely accepted that the extracellular accumulation of Aβ in senile plaques is an important event in the pathogenesis of AD.^[13] The aggregated Aβ is toxic to neurons both *in vitro* and *in vivo*^[14] and the overexpression of human amyloid precursor protein is observed in transgenic mouse models of AD,^[15] which causes neuritic plaques similar to those seen in AD patients with learning and memory deficits.^[16] The transgenic APP mice demonstrated a cognitive decline and were well accepted as very successful AD model.^[17,18] Meanwhile, soluble Aβ oligomers can inhibit cognitive function^[19] and long-term potentiation *in vitro* and *in vivo*.^[20] Moreover, the animal models where cognitive impairment and neuropathological signs are induced by intracerebral injection of pre-aggregated Aβ-oligomers are useful models for the early stages of AD,^[21] which were used as a good AD model in many studies.^[22-24]

Our results showed that the excessive deposition of Aβ caused apoptosis in hippocampus neurons, resulting in impairment of memory and learning ability in a rat model of AD. In the interventional groups, HBO/EGB 761 treatment significantly

improved the memory and cognitive capacities of AD rats, and they reduced apoptosis in hippocampus neurons. In this respect, HBO and EGB 761 had a synergistic effect. Moreover, we observed that HBO/EGB 761 treatment activated NF- κ B pathway in hippocampus neurons. These data was in line with previously published results showing that the activation of NF- κ B pathway may have a protective role in AD.^[25]

Several studies have demonstrated a correlational link between NF- κ B activity and neurodegenerative diseases such as AD, PD, and HD. They proposed that NF- κ B activation in neurons had a neuroprotective role in the degenerative process of these diseases.^[26] In addition, NF- κ B may be involved in the gene long-term memory changing from short-term memory coming from the synaptic signals, which have an important role in signal transduction, memory formation, and neural modeling.^[27] Meanwhile, in the pathological conditions, the activation of NF- κ B promotes survival neuron survival. For example, NF- κ B inhibits apoptosis through regulating apoptosis protein (IAPs), anti-oxidative enzymes and inhibition of mitochondrial depolarization, membrane permeability changes, and cytochrome C release.^[28] However, there are also evidences indicating that it may cause neuronal death. In TG2576 transgenic mice, the increased NF- κ B activation and apoptosis was found in the same cells, implying that NF- κ B activation may increase neuronal apoptosis.^[29]

Taken together, these findings demonstrated that HBO/EGB 761 ameliorates the cognitive and memory impairment in rat model of AD. The protective effects of HBO/EGB 761 are associated with a reduced apoptosis and NF- κ B pathway activation in hippocampus neurons.

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Conflicts of interest

There are no conflicts of interest.

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