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Research article

Neuroprotective effect of combined therapy with hyperbaric oxygen and madopar on 6-hydroxydopamine-induced Parkinson's disease in rats

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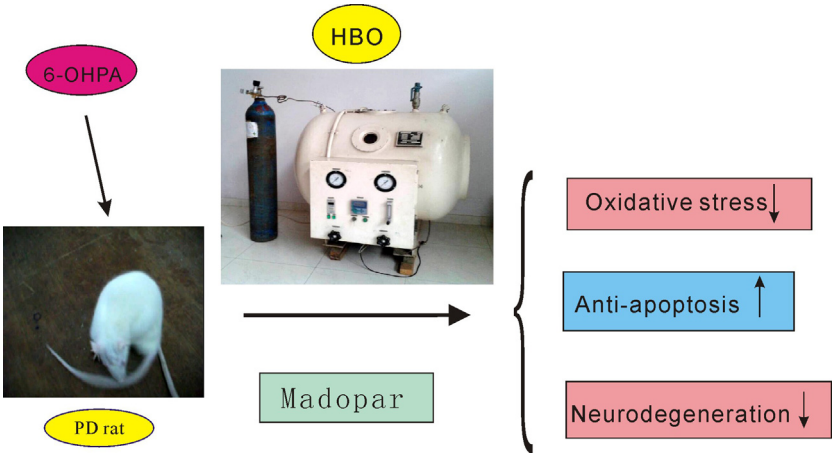
HIGHLIGHTS

- The combination therapy attenuated apomorphine-induced turns in PD rats.
- The combination therapy reduced oxidative stress and modulated the level of anti-apoptosis.
- The combination inhibited neurodegeneration of PD rats.

GRAPHICAL ABSTRACT



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ABSTRACT

Parkinson's disease (PD) is a common movement disorder in the elderly. In the present study, we examined whether the combination of hyperbaric oxygen (HBO) and madopar therapy provided a neuroprotective effect on dopaminergic neurons in the substantia nigra using a rat model of PD. Rotational assessments revealed that both HBO and combination therapy significantly attenuated apomorphine-induced turning in PD rats. Our results indicated that the combination therapy increased glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities and reduced the malondialdehyde (MDA) content in the SN. Furthermore, the combination therapy resulted in significant protection against the loss of neurons, and specifically tyrosine hydroxylase (TH)-positive neurons, in the SN and also alleviated the production of glial fibrillary acidic protein (GFAP). The levels of Bcl-2 were increased and Bax were decreased following the HBO or combination therapy. In brief, the neuroprotective effect of combined therapy with HBO and madopar against 6-OHDA-induced PD rats may rely on its ability to reduce oxidative stress and protect against Bax/Bcl-2-mediated apoptosis.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized by resting tremor, rigidity and

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bradykinesia and is a common movement disorder in the elderly [13,20]. The conventional therapy using levodopa and dopamine agonists for PD focuses primarily on relieving the motor symptoms, but this therapy does not prevent dopaminergic neuron degeneration [8,25]. Thus, there is a great demand for novel therapies that prevent neuronal death. Increasing evidence suggests that one crucial factor in the pathogenesis of PD is oxidative stress, which represents the physiological response to reactive oxygen species (ROS) production [18] in PD patients. Increased ROS production and an imbalance of the antioxidant defense and repair mechanisms lead to the loss or apoptosis of dopaminergic neurons in the substantia nigra (SN) [4]. Thus, the reversion of cellular oxidative damage represents an effective strategy for PD treatment [11].

Hyperbaric oxygen (HBO) therapy is a unique method used for the treatment of various illnesses and clinical conditions, such as carbon monoxide poisoning, cerebral ischemia, and even PD [3,35]. Increasing evidence suggests that superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase activity are increased after repeated HBO treatments [9,10], although some reports show that HBO treatment may cause excessive generation of ROS, which induces neuronal cell damage [2]. On the other hand, HBO therapy attenuates hypoxia in affected tissues, which is helpful for the protection of neuronal function. There is a great deal of recent evidence indicating that HBO therapy prevents neuronal damage and improves neurological outcome after brain ischemia [26,33]. Therefore, in our current study, 6-hydroxydopamine hydrochloride (6-OHDA)-lesioned rats were used as a model of PD to explore the protective effects of combined therapy with HBO and madopar on 6-OHDA-induced behavioral, biochemical, and pathological changes and to determine the underlying mechanisms by which the combination therapy modulates the hemi-parkinsonian factors involved in neuronal degeneration and oxidative stress.

2. Materials and methods

2.1. Animals

A total of 85 male Wistar rats weighing 250–290 g were obtained from the Experimental Animal Center of Guangxi Medical University [SYXK 2009-0002] and were allowed to acclimate in quarantine for one week prior to experimentation. The experimental animals were treated according to the Guidance Suggestions for the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of the People's Republic of China.

2.2. Unilateral 6-OHDA lesion

Surgery was performed as previously described [24], with minor modifications. Briefly, after assessing their preoperative rotation behavior to confirm that there were no abnormal rats, the rats were anesthetized using sodium pentobarbital (30 mg/kg, i.p.). Then, the rats were fixed in a stereotaxic frame. According to the atlas from [28], the coordinates of the right caudate putamen (CPu) are located 0.2 mm anterior to Bregma, 3.0 mm lateral to the sagittal suture, and 5.5 mm below the surface of the skull. Next, each animal, except for those in the sham group (injected with 10 μL of 0.2% ascorbate–saline solution), was injected with 10 μL of fresh 6-OHDA solution (diluted in 0.2% ascorbate–saline solution; final concentration of 2.0 g/L) into the CPu using a microinjector (Shanghai, China). One week later, the rats were injected with the same dose of 6-OHDA into the same location again. Two weeks later, the rats were subcutaneously injected with apomorphine (0.1 mg/kg) to induce rotation to the left side [7]. The rats exhibiting contralateral rotations faster than 7 rotations per min were considered as valid models of PD.

2.3. Study design

The qualified PD model rats (65.75%) were randomly separated into 4 groups (Groups B–E) containing 12 rats per group. Group B served as the model group, and was intragastrically administered normal saline once daily for 14 consecutive days. Group C was intragastrically given madopar (L-DOPA + benserazide, 25 + 6.25 mg/kg) for 14 days [15]. Group D received HBO treatment. Besides HBO, Group E was given madopar (the same dose of group C) treatment. Group A served as the sham group, and was intragastrically administered the same volume of normal saline as group B. The rotational behavior of the animals was tested again with apomorphine (0.1 mg/kg, s.c.) after 14 days of treatment (Fig. 1). The data are presented as the total number of rotations over 30 min.

2.4. Hyperbaric therapies

The animals were placed in hyperbaric chambers as previously described [10]. Then, the pressure was increased to 0.25 MPa at a rate of 100 kPa per min, and each treatment lasted for 60 min. Decompression was performed at a uniform rate over 10 min. HBO treatment was performed once daily for consecutive 14 days.

2.5. Measurement of SOD, GSH-Px and MDA levels in SN tissue homogenates

At the end of the experiment, six rats from each group were anesthetized using sodium pentobarbital (30 mg/kg, i.p.), and their brains were then removed. According to the rat brain stereotaxic coordinates [28], the right substantia nigra (SN) was isolated and homogenized in ice-cold physiological saline (10% w/v) to produce a 5% homogenate. The contents of SOD, GSH-Px and MDA were determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, China).

2.6. Histological and immunohistochemical examination of SN tissue

After 14 days of treatment, the remaining six rats from each group were anesthetized using sodium pentobarbital (30 mg/kg, i.p.) and subjected to a thoracotomy. The animals were rapidly perfused using 250 mL of normal saline followed by 250 mL of 4% paraformaldehyde at 4 °C to remove any remaining blood in the brain. The right SN samples were isolated, processed by routine histology procedures, embedded in paraffin, cut into 4 μm-thick section and mounted on the slide. The samples were stained with hematoxylin and eosin (HE) for histopathological examination or incubated with different antibodies for immunohistochemical examination.

According to the method of previous study [21,22,34], the waxed specimens were incubated with primary antibody (tyrosine hydroxylase (TH) antibody, or GFAP antibody, or Bax antibody, or Bcl-2 antibody, 1:100 dilution, Zhongshan Goldenbridge Biological Technology, Beijing, China) overnight at 4 °C, washed three times with 0.1 mol/L PBS for 3 min each, and incubated with biotinylated goat antirabbit immunoglobulin G (Zhongshan Goldenbridge Biological Technology, Beijing, China) at 30 °C for 25 min. After washing three times with 0.1 mol/L PBS for 3 min each, the specimens were incubated with a streptavidin–biotin complex at 30 °C for 20 min. Then, they were rinsed five times in 0.1 mol/L PBS for 3 min, incubated with diaminobenzidine for 15 min at room temperature, counterstained with hematoxylin, cleared, mounted and examined.

Cell counts and the density of GFAP-immunopositive fibers were analyzed in five independent sections each from all of the experimental groups and quantified using a microscope at a magnification

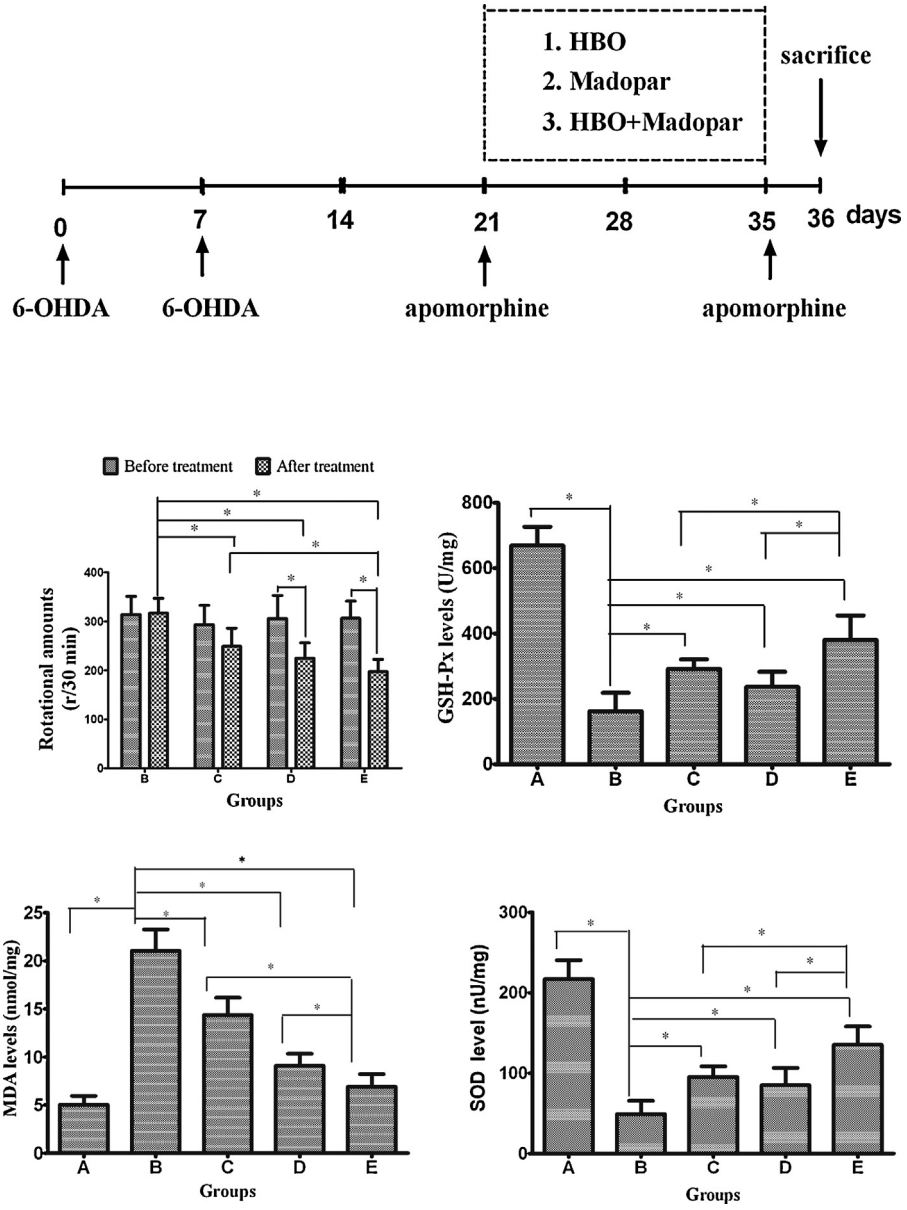

Fig. 1. Illustration of experimental procedure, evaluation of apomorphine-induced rotation and measurement of SOD, GSH-Px and MDA levels in the right SN. (A) sham group; (B) model group; (C) madopar group (L-DOPA + benserazide, 25 + 6.25 mg/kg); (D) HBO group (O_2, 0.25 MPa, 60 min); (E) HBO + madopar group. Following apomorphine injection, the number of contralateral rotations were counted for 30 min and analyzed using two-way analysis of variance (ANOVA). The remaining data were analyzed using one-way ANOVA followed by Tukey–Kramer's post hoc test. The results are presented as the means ± S.E. Note: $^{*}P < 0.05$ for this and all subsequent figures.

of 400× (Olympus, Germany). The region of interest was captured using a camera and analyzed using Imagepro-Plus software. The results of GFAP are expressed as average optical density.

2.7. Statistical analysis

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., USA). The total numbers of rotations were performed using two-way analysis of variance (ANOVA), with treatment as between subject variable and time (before treatment and after treatment) as within subject variable. The remaining data were analyzed by one-way ANOVA followed by Tukey–Kramer's post hoc test. Data are expressed as the mean ± S.E. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Assessment of apomorphine-induced rotation

Rats in the sham group without 6-OHDA lesions, apomorphine did not induce rotations (rotational amount = 0.0).

Two weeks after injection of 6-OHDA, rats in the model group exhibited a pronounced increase in rotational behavior after apomorphine administration that was greater than that of the sham group ($P < 0.05$). Following madopar, HBO or HBO plus madopar treatment, the number of rotations was reduced compared to the model group ($P < 0.05$). Compared with before treatment, the animals treated with HBO alone or combination therapy exhibited a significant decrease in rotations ($P < 0.05$) (Fig. 1). Compared with madopar treatment, the animals treated with HBO plus madopar also displayed reduced rotational behavior ($P < 0.05$).

3.2. Antioxidant enzyme and lipid peroxidation Levels in the SN

Hemi-parkinsonian rats induced by 6-OHDA, the model group, exhibited a significant reduction in SOD and GSH-Px activities in the SN compared to those in the sham group ($P < 0.05$). However, the SOD and GSH-Px activities in the SN were clearly increased due to madopar or HBO plus madopar treatment ($P < 0.05$) (Fig. 1). The localization of radical formation resulting in lipid peroxidation, measured as MDA in the rat SN tissue homogenate, is presented in Fig. 1. The MDA content in the SN tissue homogenate was increased in the model group compared to the sham group ($P < 0.05$). Interestingly, these alternations in 6-OHDA lesions rats were reversed following the madopar, HBO or HBO plus madopar treatment ($P < 0.05$).

3.3. Histopathological changes in the SN

The number of neurons in the SN was significantly decreased in rats subjected to 6-OHDA injection compared to rats not receiving the 6-OHDA injection ($P < 0.05$), while the number of neurons in the SN of rats subjected to HBO plus madopar treatment was significantly increased compared to the model group ($P < 0.05$) (Fig. 2).

3.4. TH expression in the SN

Representative microphotographs of TH immunostaining in the SN are presented in Fig. 2. The bodies and fibers of dopaminergic cells displayed intense staining and apparently immunopositive processes. The number of dopaminergic neurons was notably reduced in the model group, in which the loss of TH-positive neurons was greater than that of sham group ($P < 0.05$). Compared to the model group, the loss of neurons was inhibited by HBO plus madopar treatment, accompanied by a concomitant increase in TH-positive neurons ($P < 0.05$).

3.5. GFAP expression in the SN

Fig. 2 displays the immunohistochemical staining of GFAP-positive astrocytes. The administration of 6-OHDA caused increased GFAP expression in the SN compared to the sham group ($P < 0.05$). However, these alterations in 6-OHDA-lesioned rats were alleviated by treatment with HBO or HBO plus madopar ($P < 0.05$).

3.6. Bax and Bcl-2 expression in the SN

Immunohistological examination of the SN revealed that administration of 6-OHDA significantly down-regulated the expression Bcl-2 and up-regulated the expression of Bax in the SN of the model group ($P < 0.05$). Both HBO and HBO plus madopar treatment clearly alleviated these 6-OHDA-induced alterations ($P < 0.05$) (Fig. 2).

4. Discussion

The present study demonstrated for the first time that the combination of HBO plus madopar therapy significantly increased antioxidant capacity and reduced neurodegeneration in 6-OHDA-induced PD rats. In addition to the oxidative parameters, we clearly demonstrated changes in neurons and in the expression levels of apoptosis-related proteins in the SN of the ipsilateral hemispheres.

Currently, the partial lateral 6-OHDA-induced PD model is one of the most commonly used neurotoxin-mediated models of PD to assess motor impairments [22,31]. Stereotaxic injection of 6-OHDA into the SN, the striatum, or the medial forebrain bundle, in neonatal rats induces excessive oxidative stress and degeneration of the nigrostriatal pathway [19], the production of hydroxyl radicals during autoxidation [12,30] and the inhibition of complex I [17]. The contralateral rotation behavior, as a representative of motor dysfunction, is induced using apomorphine. This behavior reflects an upregulation of striatal dopaminergic receptors in the lesioned site due to dopamine depletion [32]. In this study, the PD model rats exhibit increased rotational behavior accompanied by a reduced number of dopaminergic neurons ($P < 0.05$). Interestingly, our results revealed that HBO plus madopar treatment significantly decreased apomorphine-induced rotational behavior, as well as oxidative stress and damage to dopaminergic neurons in the SN of 6-OHDA-induced PD rats ($P < 0.05$). Moreover, the change of rotations and the levels of MDA, GFAP, Bax and Bcl-2 in 6-OHDA-lesioned rats were reversed following the HBO alone treatment ($P < 0.05$).

The roles of SOD and GSH-Px in the protection of cells against oxidative stress and other xenobiotic compounds have been well established [29]. GSH-Px is a broadly expressed enzyme in vivo that catalyzes the decomposition of H_2O_2. SOD is an oxygen free radical scavenger. The MDA content indirectly reflects the severity of damage induced by oxygen free radicals. It was found that the activity levels of these free radical scavenging enzymes were decreased and the amount of lipid peroxidation was increased during the development of PD [37], which was associated with the ROS cascade lesions in the SN. Some studies in rats revealed that after HBO exposure at 2.5–3 ATA, oxidative stress markers, including the activity of SOD, GSH-Px, and NO, were elevated in the brain [5]. Our present results revealed that the activities of GSH-Px and SOD were significantly decreased and the MDA content was increased in valid PD model, demonstrating that oxidative stress injury was involved in the parkinsonism. Here, we found that madopar or combination therapy alleviated all these changes in 6-OHDA-lesioned rats. Whereas, HBO treatment restored MDA level. Our study suggested that HBO plus madopar treatment increases antioxidant enzyme activities while inhibiting the lipid peroxidation response. Therefore, the combination therapy effectively attenuates oxidative stress in PD.

It has also been reported that 6-OHDA-induced degeneration of the nigrostriatal pathway is paralleled by DA depletion [16]. TH is a rate-limiting enzyme that mediates the synthesis of the neurotransmitter DA. Due to a lack of dopamine beta-hydroxylase, TH-positive neurons in the midbrain are DA neurons [14]. In this study, histopathological and immunohistochemical examination of SN tissue revealed that 6-OHDA-induced lesions were associated with a massive loss of neuronal dopamine, an approximately 4-fold decrease in the total number of neurons and TH-positive neurons in the lesioned SN compared to the sham group. HBO plus madopar treatment treatment at 0.25 MPa for 60 min protected against the loss of total neurons and TH-positive dopaminergic neurons in the SN. Therefore, consistent with previous findings [23], our results demonstrated the neuroprotective effects of combination therapy against the 6-OHDA-induced degeneration of the nigrostriatal dopaminergic pathway. In addition, according to the previous studies [1,6], the density of GFAP-immunopositive fibers in the SN was increased in the model group. However, the density of GFAP-immunopositive fibers was decreased under HBO alone or HBO plus madopar treatment.

Another mechanism that could be involved in the neuroprotective effect of HBO therapy is its anti-apoptotic function. It was previously shown HBO attenuate apoptosis in an ischemic wound model [36]. Recently, it was found that that HBO combined with anti-parkinson granule therapy protected neurons against apoptosis in parkinsonian rats induced using 6-OHDA [27]. In the present study, we measured the expression of the apoptosis-related factor Bax and the anti-apoptosis-related factor Bcl-2. We found that the expression levels of Bax and Bcl-2 were increased 4.6-fold and decreased 3.8-fold, respectively, in the 6-OHDA-lesioned SN compared to the sham group. Strikingly, HBO or HBO plus madopar

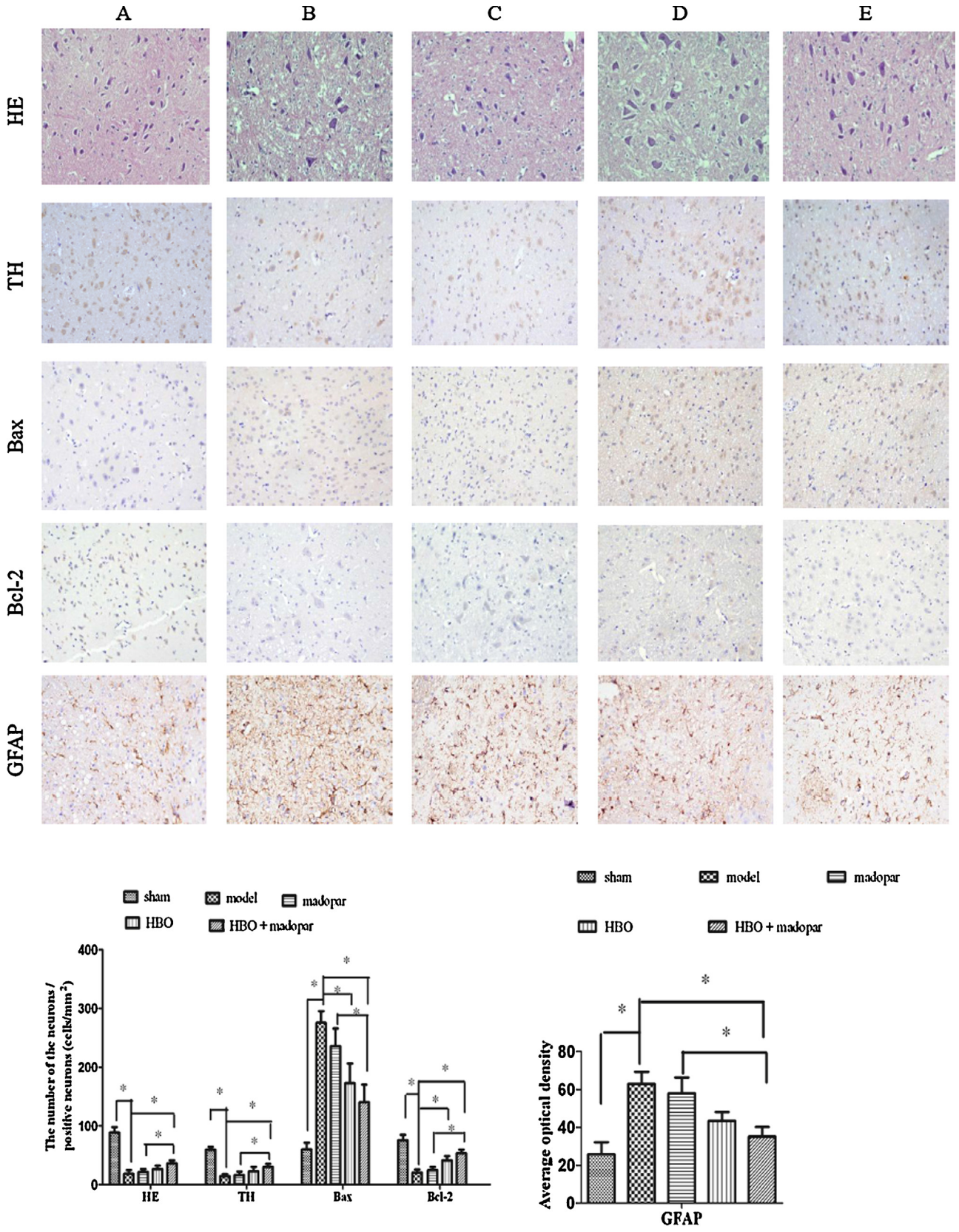

Fig. 2. Effect of HBO treatment on histopathological and immunohistochemical changes in the SN of 6-OHDA-induced PD rats. Upper: representative photographs displaying the number of the neurons (HE, TH, Bax, and Bcl-2) and the density of GFAP-immunopositive fibers (GFAP). (A) sham group; (B) model group; (C) madopar group (L-DOPA + benserazide, 25 + 6.25 mg/kg); (D) HBO group (O_2, 0.25 MPa, 60 min); (E) HBO + madopar group. Lower: calculation of the number of neurons (HE, cells/mm^2), positive neurons (TH, Bax, and Bcl-2; cells/mm^2) and the density of GFAP-immunopositive fibers (GFAP). Each column represents the means ± S.E. ($n = 6$). Scale bar = 200 μm. Statistical analyses were performed using one-way ANOVA followed by Tukey–Kramer's post hoc test.

treatment promoted Bcl-2 expression and inhibited Bax expression in the PD model rats. Thus, the anti-apoptotic function of HBO or the combination therapy may be partially responsible for its neuroprotective effect.

In conclusion, our results demonstrate a significant neuroprotective effect of HBO plus madopar therapy in 6-OHDA-induced PD rats. The most important findings in the present study include the prevention of the loss of TH-positive neurons and the improvement in behavioral activity with the combination therapy. In addition to these results, HBO or HBO plus madopar treatment was found to increase the level of Bax and decrease the level of Bcl-2 in the SN of PD rats. Thus, these findings suggest that HBO plus madopar therapy represents an attractive alternative for the treatment of PD.

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