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Anti-inflammatory and anti-apoptotic effects of hyperbaric oxygen preconditioning in a rat model of cisplatin-induced peripheral neuropathy

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ABSTRACT

Objective(s): Cisplatin-induced peripheral neuropathy is a debilitating side effect in patients receiving this drug. Recent studies suggest hyperbaric oxygen (HBO) therapy as a new treatment approach for models of neural injury. The aim of the current study was to determine the protective effects of HBO preconditioning against peripheral neuropathy induced by Cisplatin (CDDP).

Materials and Methods: The present study was conducted on 4 groups of rats: Sham group; HBO group (60 min/d); Control group (CDDP 2 mg/kg/d); Precondition group (HBO+CDDP). Mechanical threshold testing was weekly carried out using von Frey filament. Sciatic nerve and associated ganglia were removed five weeks after the first CDDP injection for biochemical evaluation of malondialdehyde (MDA) content and myeloperoxidase (MPO) activity, immunohistochemistry of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), TNF-α, caspase-3 and iNOS, and transmission electron microscopic (TEM) assessments.

Results: MDA levels and MPO activities were significantly decreased in preconditioned rats. Attenuated TUNEL reaction along with attenuated caspase-3, TNF-α, and iNOS expression could be significantly detected in preconditioned rats. Also, HBO preconditioning improved the nociceptive threshold.

Conclusion: The results suggest that HBO preconditioning can attenuate peripheral neuropathy caused by cisplatin in rats.

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Introduction

Chemotherapy-induced peripheral neurotoxicity (CIPN) as a common debilitating side effect of antineoplastic drugs is a major clinical problem in cancer patients that reduces the quality of life and restricts continuity of treatment (1). Among the antineoplastic agents, cisplatin (cis-diamminedichloroplatinum II; CDDP) is strongly neurotoxic causing disabling peripheral neuropathies, clinically characterized by distal paresthesis and sensory ataxia (2). In this regard, it is well known that formation and accumulation of cisplatin-DNA adducts with guanine-guanine intrastrand cross-link in dorsal root ganglion (DRG) sensory neurons and glial cells triggers the apoptotic process, which can lead to secondary nerve fiber axonopathy and partial degeneration of myelinated axons in parallel to reduced conduction velocity of the sensory nerves (3-5). Meanwhile, another study has shown that covalently bindings of cisplatin to mitochondrial DNA (mtDNA), along with binding to nuclear DNA (nDNA), resulted in mtDNA damage and established a distinct mechanism for neurotoxicity induced by cisplatin (6). It also emphasized the role of oxidative stress in CDDP activated mitochondrial apoptotic pathway (7). Therefore, it has been postulated that decreased CDDP-induced DNA adducts or increased resistance of neural cells to neurotoxicity of CDDP may offer some protection against the sensory peripheral neuropathy. However, no effective methods have been suggested for prevention or treatment of cisplatin-induced neurotoxicity so far, but some efforts have been made to reduce CDDP-neurotoxic effects such as the simultaneous use of neuroprotective compounds along with cisplatin (8, 9).

Treatment with hyperbaric oxygen, 100% oxygen at a pressure upper than sea level, is suggested as one of these methods. In this regard, it is well documented that HBO has neuroprotective effects against spinal cord and brain injuries (10-13), neurodegenerative disorders (14, 15), and peripheral nerve injuries (16, 17). Accumulating evidence indicates an association between the beneficial effects of HBO to a variety of biological properties mainly anti-oxidative (18, 19), anti-inflammatory (20, 21), and anti-apoptotic (22, 23) properties, in addition to improvement of oxygen supply

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and neural metabolism (24, 25). Also, only a few studies have been done on the effects of HBO therapy against cisplatin-induced nephrotoxicity by modification of the oxidant/antioxidant system (26, 27) and against cisplatin-induced ototoxicity without mentioning the mechanism (28, 29). Despite some evidence for the neuroprotective effects of HBO in various experimental neural damage, there have been no studies on the effect of HBO on cisplatin-induced peripheral neuropathy. According to this, we examined the neuroprotective effects of HBO on neural cell apoptosis, inflammation, and myelin degeneration induced by cisplatin in rats.

Materials and Methods

Animals

Male adult Wistar rats were used (275–300 g) (Laboratory Animal Research Center, Sari, Iran) in this study. The rats were kept under constant conditions of lighting (12 hr light/dark cycle) and temperature (23±1 ˚C) before and throughout the experiment. All procedures were performed in accordance with the guidelines of the university's animal care codes (IR. MAZUMS..REC.1397.2954).

Induction of neuropathy and experimental design

Peripheral neuropathy was induced with an intraperitoneal injection of 2 mg/kg cisplatin (Ribosepharm Company) twice a week for four weeks (30, 31). The rats were housed in the HBO chamber; the pressure was gradually increased, maintained in 2 atmosphere absolute (ATA), and then allowed to breathe 100% oxygen for 60 min a day (17, 32).

In the pilot study, three modes of HBO treatment were used, namely, Curative group (received CDDP for 4 weeks and then after the final injection allowed to breath HBO 60 min/d for 7 days), Preventive group (allowed to breath HBO 60 min/d immediately after each CDDP injection for 7 days), and Precondition group (allowed to breath HBO 60 min/d, preconditioned for 7 days and then received CDDP for 4 weeks). Mechanical nociceptive threshold testing only showed improvement in the precondition group. Therefore, the study was followed only in HBO preconditioning. Accordingly, the animals were randomly divided into four groups: (I) Sham group (received saline alone, IP as a volume of CDDP, n=10); (II) HBO group (allowed to breath HBO 60 min/d for 7 days and then received saline, IP as a volume of CDDP, n=10); (III) Control group (received CDDP 2mg/kg/d, IP, twice a week for 4 weeks, n=10); (IV) Precondition group (allowed to breath HBO 60 min/d, preconditioned for 7 days and then received CDDP for 4 weeks, n=10). The doses and treatment plans were based on previous experiments (17, 30-32).

Nociception assay

Mechanical nociceptive threshold was measured weekly in all groups by examining the hind paw withdrawal response to von Frey filaments stimulation (33) until the end of the experiment. The animals were housed in a plastic cage, and a series of calibrated von Frey filaments were used perpendicularly to the animal's hind paw mid-plantar surface. The clear paw withdrawal was defined as a positive response. Four weeks after the first CDDP injection, the rats were euthanized with sodium pentobarbital and then both sciatic nerves and related DRG were harvested for biochemical and immunohistochemical evaluations.

Biochemistry

The obtained sciatic nerve samples (right side) were stored in a -80 ˚C freezer until homogenization for evaluation of tissue malondialdehyde (MDA) levels (34) as a product of lipid peroxidation and for evaluation of tissue myeloperoxidase (MPO) activity (35) as an indicator of polymorphonuclear leukocyte accumulation.

Light and electron microscopy

The obtained sciatic nerve samples (right side) were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide and finally embedded in epoxy resin. For light microscopic assessments, 1 µm thick (semi-thin) sections were stained with toluidine blue. For transmission electron microscopic (TEM) assessments, 100 nanometers thick (ultra-thin) sections were stained with uranyl acetate and lead citrate. Average G-ratio (quotient axon diameter/fiber diameter as an indicator of the rate of myelination) was measured in semi-thin sections, as previously described (36). Finally, these were analyzed using the ImageJ software package (MacBiophotonics ImageJ 1.41a).

TUNEL staining

TUNEL staining was performed with a TUNEL detection kit (Roche). After rehydration, the sections were incubated for 10 min in 3% H2O2, for 15 min in proteinase-K, for 60 min in TUNEL reaction mixture, and for 30 min in converter POD at 37 ˚C. Finally, the sections demonstrated for 5 min with DAB and counterstained with hematoxylin. For quantitative analysis, immunohistochemical photographs (n=5 photos from each sample) were evaluated through densitometry using the ImageJ software package (MacBiophotonics ImageJ 1.41a).

Immunohistochemistry

For immunohistochemistry, 5-µm thick sections were prepared from each formalin-fixed paraffin-embedded block (left side). The sections were incubated in anti-caspase 3 rabbit polyclonal antibody (1:50 in PBS, v/v, Abcam), anti-TNF-α rabbit polyclonal antibody (1:50 in PBS, v/v, Elabscience), or anti-iNOS rabbit polyclonal antibody (1:50 in PBS, v/v, Abcam) at 4 ˚C for 24 hr. Finally, the sections were incubated for 2 hr with secondary antibody (goat anti-rabbit IgG, Elabscience) and detected by DAB for 10 min. For quantitative analysis, immunohistochemical photographs (n=five photos from each sample) were evaluated through densitometry using the ImageJ software package (MacBiophotonics ImageJ 1.41a).

Statistical analysis

Statistical analysis was performed using SPSS software (Ver. 16), and the results were presented as mean values (±SD). Kruskal-Wallis test and one-way analysis of variance were applied to compare data between the groups. The *P-value*<0.05 was considered statistically significant.

Table 1. Effects of hyperbaric oxygen (HBO) preconditioning on mechanical allodynia (Von Frey assay)

Group	Time (week)				
	0 W	1 W	2 W	3 W	4 W
Sham	54.33±13.88	54.33±13.88	51.50±17.00	54.33±13.88	54.33±13.88
HBO	51.50±17.00	51.50±17.00	54.33±13.88	48.67±11.63	51.50±17.00
Control	53.20±15.21	12.17±3.18***	12.17±3.18***	13.05±3.16***	12.60±3.36***
Precondition	51.50±17.00	17.83±3.61	20.50±3.73##	22.33±2.68##	24.17±2.49###

Data are represented as mean±SD of rats/group. ***P<0.001 vs sham and HBO groups; ##P<0.01 vs control group; ###P<0.001 vs control group

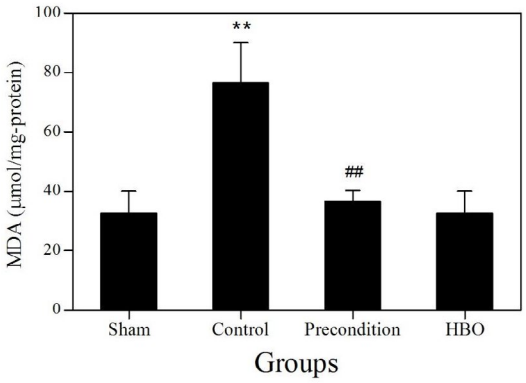

Figure 1. Effects of hyperbaric oxygen (HBO) on the malondialdehyde (MDA) level
Histogram shows the levels of MDA in the sciatic nerve at the end of the experiment. MDA levels significantly increased after CDDP injection in control group. While this elevation significantly attenuated with HBO treatment in precondition group. Values are expressed as micromoles per mg of protein (µmol/mg-protein). **P<0.01 vs sham and HBO groups; ##P<0.01 vs control group
MDA: malondialdehyde; CDDP: cisplatin

Results

Nociceptive response

The Mechanical nociceptive rating scores of all groups as mean value±SD have been presented in Table 1. Sensitivity to mechanical stimulus was significantly (P<0.001) increased following CDDP injection compared with sham and HBO groups. At the end of the fourth week, a significant difference (P<0.001) in the nociceptive score was observed between precondition and control groups.

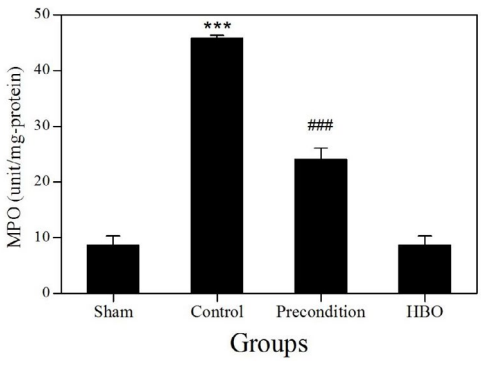

Figure 2. Effects of hyperbaric oxygen (HBO) on myeloperoxidase (MPO) activity
Histogram shows the levels of MPO activity in the sciatic nerve at the end of the experiment. MPO activities significantly increased after CDDP injection in the control group. While this elevation significantly attenuated with HBO treatment in precondition group. Values are expressed as unit per mg of protein (unit/mg-protein). ***P<0.001 vs sham and HBO groups; ###P<0.001 vs control group
MPO: myeloperoxidase; CDDP: cisplatin

Biochemical analysis

Figure 1 shows the MDA levels for all groups at the end of the study. CDDP injection in the control group significantly (P<0.01) increased the lipid peroxidation level compared with sham and HBO groups. While the level of MDA in the precondition group was significantly (P<0.01) lower than the control group.

Figure 2 shows the MPO activities for all groups at the end of the study. CDDP injection in the control group significantly (P<0.001) increased the MPO activities compared with sham and HBO groups. While the level of MPO activity in the precondition group was significantly (P<0.001) lower than the control group.

Histopathologic assessment

TEM photographs of the control group revealed a limited myelin sheath degeneration in the sciatic nerve (Figure 3C). Treatment with HBO in precondition group reduced the extent of demyelination; so that normal microscopic appearance was detected in some of the nerve fibers (Figure 3D). While there was no detectable damage in sham (Figure 3A) and HBO (Figure 3B) groups.

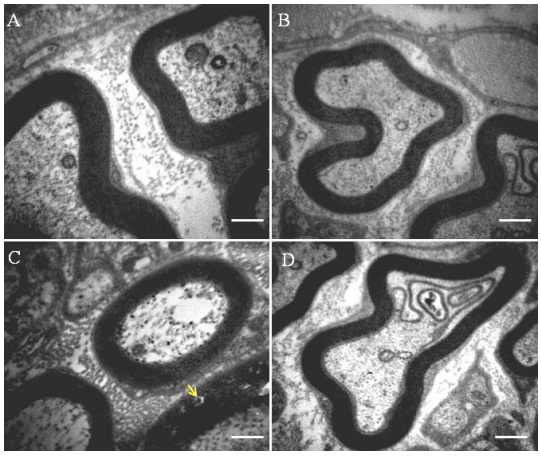

Figure 3. TEM photomicrograph of the transverse sciatic nerve sections shows limited myelin degeneration (arrow) after CDDP injection in control group (3C), while sections of precondition group (3D) show the reduced demyelination. No detectable myelin degeneration was shown in sham (3A) and HBO (3B) groups. Stained with uranyl acetate and lead citrate; scale bar=2 µm
TEM: transmission electron microscopic; CDDP: cisplatin; HBO: hyperbaric oxygen

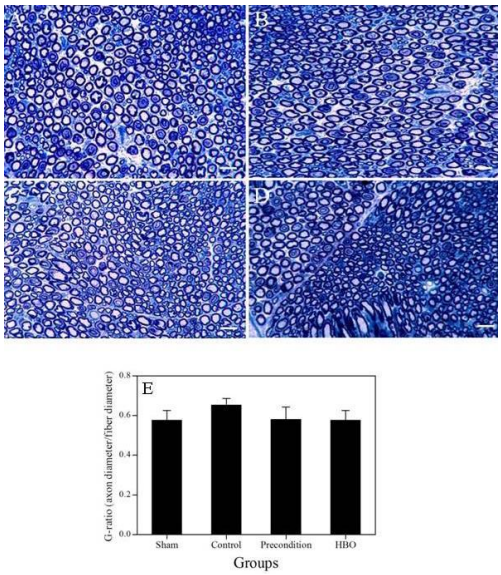

Figure 4. Light photomicrographs of the semi-thin cross-sections of sciatic nerves in sham (3A), hyperbaric oxygen (HBO)(3B), control (3C), and precondition (3D) groups (stained with toluidine blue; scale bar=100 µm). Histogram shows G-ratio (quotient axon diameter/fiber diameter) analysis of the myelinated nerve fibers in all groups (4E). There are no significant differences between the groups (P>0.05)

Figure 4E shows the histogram of the quantitative analysis of myelin thickness by G-ratio in semi-thin cross-sections of experimental groups (4A: sham; 4B: HBO; 4C: control; 4D: precondition). CDDP injection in the control group did not result in significant changes in the G-ratio compared with sham and HBO groups (P>0.05).

TUNEL assessment

Figure 5 shows the TUNEL reaction in the DRG of experimental groups. Many cells in the DRG of the control group intensely reacted with tunnel (5C). In contrast, few TUNEL-positive cells were observed in the DRG of precondition HBO-treated rats (5D). Meanwhile, in the sham (5A) and control (5B) groups, almost no TUNEL-reaction was detected. Figure 5E shows the histogram of the quantitative analysis of TUNEL-positive staining in the experimental groups.

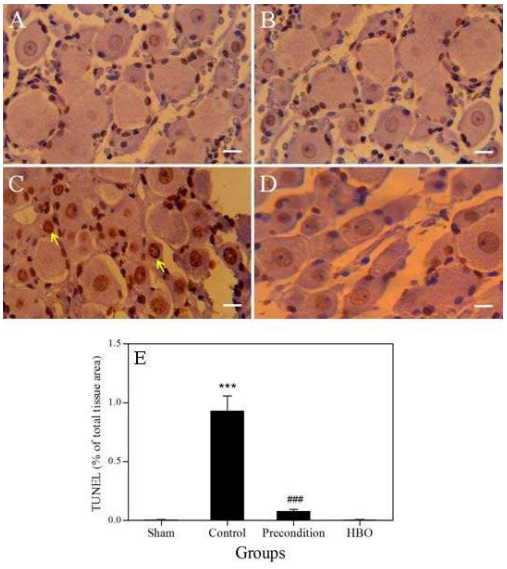

Figure 5. Light photomicrographs show TUNEL-positive cells in the dorsal root ganglion of sham (5A), HBO (5B), control (5C), and precondition (5D) groups. The positive staining of TUNEL is presented by brown color of nucleus (arrows), the remainder of TUNEL reactions were counterstained with hematoxylin (scale bar=100 µm). Densitometry analysis of photomicrographs for TUNEL reaction was assessed in all experimental groups using the ImageJ software package (5E). Data are expressed as a percentage of total tissue area. $^{***}P$<0.001 vs sham and HBO groups; $^{###}P$<0.001 vs control group
TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; HBO: hyperbaric oxygen

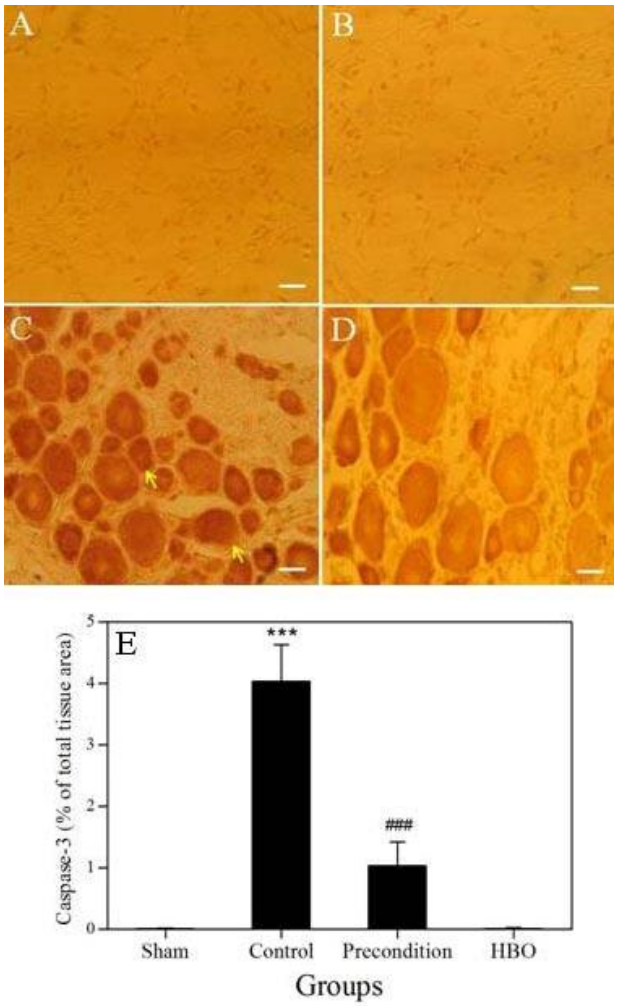

Figure 6. Light photomicrographs show immunohistochemical expression of caspase-3 in the dorsal root of sham (6A), hyperbaric oxygen (HBO)(6B), control (6C), and precondition (6D) groups. The positive staining of caspase-3 is presented by brown color of cytoplasm (arrows) (scale bar=100 µm). Densitometry analysis of immunohistochemical photomicrographs for caspase-3 was assessed in all experimental groups using the ImageJ software package (6E). Data are expressed as a percentage of total tissue area. $^{***}P$<0.001 vs sham and HBO groups; $^{###}P$<0.001 vs control group

Immunohistochemical assessment

Immunohistochemical staining of caspase-3 in the DRG of experimental groups is shown in Figure 6. Cisplatin increased caspase-3 expression in the DRG of control group (6C). HBO treatment in the precondition group reduced caspase-3 staining in DRG (6D) compared with the control group. Meanwhile, almost no detectable immunohistochemical reaction was shown in the sham (6A) and HBO (6B) groups. Figure 6E shows the histogram of the quantitative analysis of caspase-3 positive staining in the experimental groups.

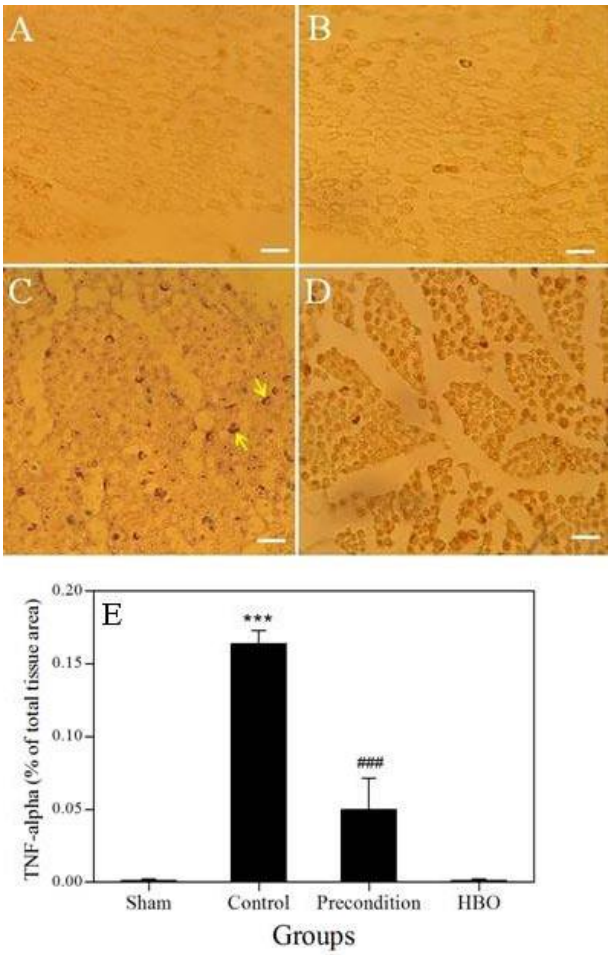

Figure 7. Light photomicrographs show immunohistochemical expression of TNF-α in the sciatic nerve of sham (7A), HBO (7B), control (7C), and precondition (7D) groups. The positive staining of TNF-α is presented by brown color of cytoplasm (arrows) (scale bar = 100 µm). Densitometry analysis of immunohistochemical photomicrographs for TNF-α was assessed in all experimental groups using the ImageJ software package (7E). Data are expressed as a percentage of total tissue area. $^{***}P<0.001$ vs sham and HBO groups; $^{###}P<0.001$ vs control group
TNF-α: tumor necrosis factor alpha; HBO: hyperbaric oxygen

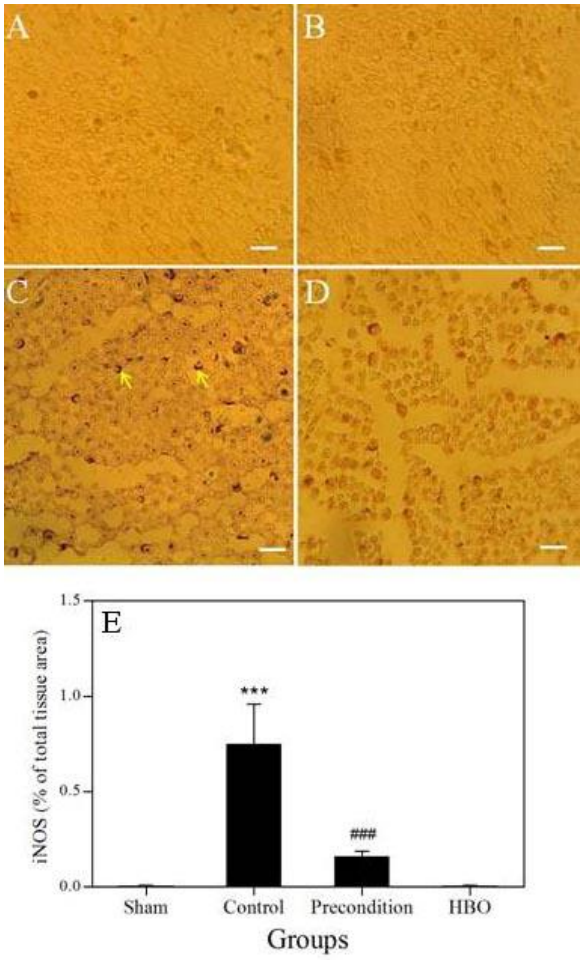

Figure 8. Light photomicrographs show immunohistochemical expression of iNOS in the sciatic nerve of sham (8A), HBO (8B), control (8C), and precondition (8D) groups. The positive staining of iNOS is presented by brown color of cytoplasm (arrows) (scale bar=100 µm). Densitometry analysis of immunohistochemical photomicrographs for iNOS was assessed in all experimental groups using the ImageJ software package (F8E). Data are expressed as a percentage of total tissue area. $^{***}P<0.001$ vs sham and HBO groups; $^{###}P<0.001$ vs control group
iNOS: inducible nitric oxide synthase; HBO: hyperbaric oxygen

Figure 7 shows the immunohistochemical staining of TNF-α in the sciatic nerve of experimental groups. Cisplatin increased TNF-α protein expression in the sciatic nerve obtained from the control group (7C). HBO treatment in the precondition group reduced TNF-α staining in sciatic nerve (7D) compared with the control group. Meanwhile, almost no detectable immunohistochemical reaction was shown in the sham (7A) and HBO (7B) groups. Figure 7E shows the histogram of the quantitative analysis of TNF-α positive staining in the experimental groups.

Immunohistochemical staining of iNOS in the sciatic nerve of experimental groups is shown in Figure 8. Cisplatin increased iNOS protein expression in the sciatic nerve obtained from control group (8C). HBO treatment in the precondition group reduced iNOS protein expression in sciatic nerve (8D) compared with the control group. Meanwhile, almost no detectable immunohistochemical reaction was shown in the sham (8A) and HBO (8B) groups. Figure 8E shows the histogram of the quantitative analysis of iNOS positive staining in the experimental groups.

Discussion

The findings of this study indicated that hyperbaric oxygen therapy attenuates apoptosis and inflammation, and improves the nociceptive threshold against cisplatin-induced peripheral neuropathy in rats.

Apoptosis of the DRG cells is a major contributing factor of secondary cisplatin-induced nerve fiber axonopathy (37). In this respect, it is well known that cisplatin-induced apoptosis is mediated through expression of pro-apoptotic indicators such as caspase-3 and Bax (6, 38) so that the use of caspase inhibitors reduces acute cisplatin-induced apoptosis in DRG neurons (6). Our immunohistochemical results indicated that caspase-3 expression considerably increased in sensory DRG neurons with CDDP injection. Meanwhile, these up-regulations significantly attenuated with HBO treatment. Studies on cerebral ischemia have shown that exposure to hyperbaric oxygen prevents apoptosis by reducing caspase-3 (39) and phosphorylated-p38 mitogen-activated protein kinase (40), and mitochondrial ATP-sensitive potassium channels opening (41). Also, HBO therapy by reduction

of adaptor molecule apoptosis-associated speck-like protein (42), caspase-3 (43), and hypoxia-inducible factor-1α (44) prevented apoptosis in experimental spinal cord injuries. Meanwhile, studies documented that hyperbaric oxygenation inhibits apoptosis in neuropathic pain induced by chronic constriction injury (32, 45, 46). Recently, our laboratory found that HBO protects the neurons against retrograde apoptosis through different mechanisms including, caspase-3 down-regulation in rat sciatic nerve transection model (17). Oxidative stress and neuroinflammation are known to be associated with cisplatin-induced peripheral neurotoxicity (47). Meanwhile, due to weak cellular antioxidant defenses, peripheral nerves are susceptible to oxidative stress (48), which is causing lipid peroxidation (49), nerve inflammation, and damage to the myelin sheath (50). Gilardini *et al.* (51) documented that CDDP injection (2 mg/kg/d, IP, twice weekly for 4 weeks) did not induce severe pathological alterations of the myelin morphology despite a decrease in nerve conduction velocity (NCV). Our present study showed limited myelin sheath degeneration in sciatic nerve after CDDP injection despite an increased sensitivity to mechanical stimulus. Also, treatment with HBO decreased malondialdehyde and myeloperoxidase in the sciatic nerve. In this regard, some studies reported the improvement of enzymatic antioxidant activity after HBO treatment. Our recent study showed that hyperbaric oxygen therapy decreased MDA level and increased SOD and CAT activities following sciatic nerve transaction (17). Repetitive HBO treatment increased significantly SOD activity and decreased MDA in a rat model of neuropathic pain (52). TNF-α is one of the inflammatory mediators that play an important role during neuroinflammation, which is involved in iNOS induction (53). Our immunohistochemical results indicated that TNF-α and iNOS expression considerably increased in sciatic nerve with CDDP injection. Meanwhile, these up-regulations significantly attenuated with HBO treatment. Studies have revealed that anti-inflammatory effect is one of the potential mechanisms of HBO neuroprotection. In this regard, it has been demonstrated that the antinociceptive effects of HBO in experimental neuropathic pain are partially associated with anti-inflammatory effects through decreasing iNOS, TNF-α, and/or IL-1ß (16, 54, 55). In the experimental model of spinal cord injury, HBO therapy attenuated NF-kB, TNF-α, and IL-1ß levels (11). Also, a study documented that HBO therapy decreased COX-2 level after cerebral ischemia (56). In addition, our recent investigation showed that HBO therapy reduces COX-2 level after sciatic nerve transection (17). Miao *et al.* recently documented that hyperbaric oxygen treatment alleviates paclitaxel-induced peripheral neuralgia through decreasing inflammatory cytokines such as tumor necrosis factor-alpha and interleukin 1 beta and inhibiting astrocyte activation in the spinal cord (57). One of the common symptoms associated with peripheral neuropathy is sensitized nociceptor response under different mechanisms such as alternation in peripheral receptor sensitization and sprouting fibers (58). The results of our present study showed a decrease in sensitivity to mechanical stimulus in HBO-treated rats; however, this reduction was significant only in the precondition group. On the other hand, Miao *et al.* found that hyperbaric oxygen treatment after onset of neuropathy significantly decreased allodynia in paclitaxel-induced peripheral neuropathy (57). HBO preconditioning indicated that HBO activates the intrinsic mechanisms involved in the protection and repair of the organs, causing resistance to insult and subsequent damage (59). Regarding the antinociceptive effect and mechanisms of action of HBO, studies documented that HBO therapy reduces neuropathic pain through the AKT/TSC2/mTOR pathway (60), activation of the NO-cGMP-PKG signaling transduction pathway (61), regulation of the Kindlin-1/Wnt-10a signaling pathway (62), and P2X4R expression (32) in peripheral nerve injuries. Also, inhibition of apoptosis and reduction of inflammatory factors by HBO therapy are involved in reducing neuropathic pain (46, 55).

Conclusion

All the findings of the present study demonstrated that hyperbaric oxygen preconditioning had protective effects against CDDP-induced peripheral neuropathy in rats.

Acknowledgment

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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