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## The effect of hyperbaric oxygen on neuroregeneration following acute thoracic spinal cord injury

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### ABSTRACT

**Aims:** Although hyperbaric oxygen (HBO) treatment following spinal cord injury (SCI) have been studied in terms of neurological function and tissue histology, there is a limited number studies on spinal cord tissue enzyme levels.

**Main methods:** The effect of HBO treatment in SCI was investigated by measuring superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), nitric oxide synthase (NOS) and nitric oxide (NO) activity in the injured tissue. SCI was induced by applying an aneurysm clip extradurally at the level of T9–T11 vertebrae. Preoperative HBO (preopHBO) treatment was applied for 5 days and postoperative HBO (postopHBO) for 7 days.

**Key findings:** In the preopHBO group, a significant decrease was observed in NOS and NO compared to the SCI group. There was a decrease in SOD, NOS and NO in the postopHBO group when compared to the SCI group. In the pre–postHBO group SOD, GPx, NOS and NO decreased significantly. There was a decrease in SOD in postopHBO compared to preopHBO. In the prepostopHBO, SOD decreased significantly compared to that in the preopHBO group. The prepostopHBO presented a significant decrease in GPx compared to postopHBO ( $p < 0.05$  for all parameters). No significant difference was observed for catalase for all groups. Significant improvement was found in BBB scores for both postopHBO and prepostopHBO groups when compared to the SCI group ( $p < 0.05$ ). **Significance:** HBO treatment was found to be beneficial following SCI in terms of biochemical parameters and functional recovery in the postoperative period.

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### Introduction

Spinal cord injury (SCI) is a serious health problem with high care costs, and social and psychological burdens (Hodgetts et al. 2009). Typically, traumatic SCI leads to functional losses due to axonal destruction and cell death (Schwab and Bartholdi, 1996). Trauma to the spinal cord causes both primary and secondary damage. The primary damage refers to the loss of spinal cord integrity due to mechanical factors. The delayed secondary damage refers to a complex array of pathophysiological processes including ischemia, edema, inflammation, excitotoxicity and oxidative cell damage (Norenberg et al. 2004, Hodgetts et al. 2009). Much of the damage is due to glutamate excitotoxicity,  $Ca^{+2}$  overload and oxidative stress (Juurink and Paterson 1998). After SCI, inflammatory cells produce highly reactive oxidizing agents that attack the molecules crucial for cell function and modify their chemical structure (Young 1995, Liu et al. 1998, Vaziri

et al. 2004). The oxidative damage occurs in many disorders ranging from slow neurodegenerative diseases like amyotrophic lateral sclerosis and Parkinson's disease to acute events like stroke and trauma. Thus, each of the mechanisms of secondary damage has been focus of intensive research (Young 1995).

SCI treatments focus on preventing the onset of edema, ischemia and tissue destruction by applying different treatments in the early period of trauma. Recent reports have claimed that hyperbaric oxygen (HBO) treatment may be beneficial in acute and chronic SCI. Although there are studies on the HBO treatment following SCI, these are mainly histological studies or studies assessing neurological function; studies on the effect of HBO on spinal cord tissue enzyme levels is rather limited. There are only two reports published so far, one by Kahraman et al. (2007) that measured thiobarbituric acid reactive substance, superoxide dismutase (SOD), glutathione peroxidase (GPx) levels, and the other study is by Topuz et al. (2010) who measured malondialdehyde, SOD, GPx and catalase levels.

In this study, we investigated the effect of HBO treatment in SCI by measuring SOD, GPx, catalase, NO (nitric oxide) and nitric oxide synthase (NOS) enzyme activity in the injured spinal cord tissue.

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## Materials and methods

Forty-eight adult male Sprague–Dawley rats were used in total. The experimental animals were divided into eight groups:

- Group I : sham (control),  $n = 6$
- Group II : spinal cord injury (SCI),  $n = 6$
- Group III : sham + preoperative HBO (preopHBO),  $n = 6$
- Group IV : SCI + preoperative HBO (preopHBO),  $n = 6$
- Group V : sham + postoperative HBO (postopHBO),  $n = 6$
- Group VI : SCI + postoperative HBO (postopHBO),  $n = 6$
- Group VII : sham + preoperative + postoperative HBO (prepostHBO),  $n = 6$
- Group VIII : SCI + preoperative and postoperative HBO (prepostHBO),  $n = 6$

Rats were anesthetized intraperitoneally with combination of 50 mg/kg ketamine HCl, 5 mg/kg xylazine HCl and 1 mg/kg acepromazine maleate. The back skin of the rats were shaved and disinfected with povidone-iodine solution. The skin, percutaneous tissues and paravertebral muscle fascia were incised longitudinally in the midline at the level of thoracic 9 (T9) to thoracic 11 (T11) vertebrae. The muscles overlying the spine were stripped laterally with an oblique dissection. A bilateral laminectomy was performed to expose the spinal cord and the clip compression injury model developed by Rivlin and Tator (1978) was performed by applying an extradural clip for 1 min (force of clip was 30 g). Sham groups were subjected to laminectomy only. Postoperative cefazolin (30 mg/kg) was given to prevent infection for five days.

The animals were handled under the prescriptions for animal care and experimentation of the relevant European Communities Council Directive (86/609/EEC) and all the procedures were approved by the Institutional Animal Ethics Committee of Ege University, Izmir, Turkey.

### Preoperative HBO before SCI

Rats received HBO at 2.80 ATA 60 min daily for 5 days starting from the fifth preoperative day. On the sixth day, all rats were operated within 1 h and sacrificed on the first postoperative day.

### Postoperative HBO after SCI

Following SCI, rats received HBO at 2.80 ATA for 60 min daily for 5 days starting from the first postoperative hour. All rats were sacrificed on the eight postoperative day.

### Preoperative + Postoperative HBO

Before SCI, rats received HBO at 2.80 ATA for 5 days starting from the fifth preoperative day. Starting from the first postoperative hour, the rats received HBO at 2.80 ATA for 60 min daily for 7 days and were sacrificed on the eight postoperative day.

For all groups, rats were deeply anesthetized with 50 mg/kg ketamine and xylazine (5 mg/kg) and decapitated. A block of spinal cord tissue extending from 2 cm proximal to 2 cm distal to the injury was removed immediately after perfusion and snap frozen at 80 °C.

Enzyme activities of SOD, GPx, catalase, NO and NOS were measured for each rat. The SOD measurement level was performed on the basis of the colorimetric determination of SOD's inhibition of the color reaction given by the superoxide radical and the results were expressed in U/mg. Catalase activity was measured using colorimetric method based on the dismutation of H<sub>2</sub>O<sub>2</sub> by catalase; absorbance was read in a spectrophotometer at 240 nm according to the method described by Aebi (1984). Oxidized glutathione that is formed by the

reduction of organic peroxides by GPx is converted into its reduced state through a reaction catalyzed by glutathione reductase. During this conversion, the NADPH (nicotinamide adenine dinucleotide phosphate) that is present in the environment is oxidized to NADP<sup>+</sup> and reduced glutathione is synthesized with the transfer of liberated hydrogen molecules to oxidized glutathione. In the course of these reactions, a time-dependent reduction is observed in the absorbance at 340 nm wavelength. The results were expressed as U/mg protein. NOS concentration was determined using Griess reaction for the colorimetric measurement of total nitrite, which is the final product of NO<sup>-</sup> formed with the catalyzing role of NOS in aqueous solutions. The results were expressed in nmol/ml/min. For the determination of NO<sup>-</sup> levels, colorimetric method based on the addition of Griess reagent on nitrite was used (Kobzik et al. 1994, Sun et al. 2003). The results were expressed as μM/mg.

The motor function of rats was assessed using the BBB locomotor rating scale (Basso et al., 1995).

### Statistical analysis

SPSS (Statistical Package for Social Sciences) for Windows 17.0 was used for statistical analysis. Kruskal–Wallis test was used for the comparison of quantitative data among three or more and Mann–Whitney *U* test for comparing two experimental groups.

## Results

### Biochemical effects of HBO treatment

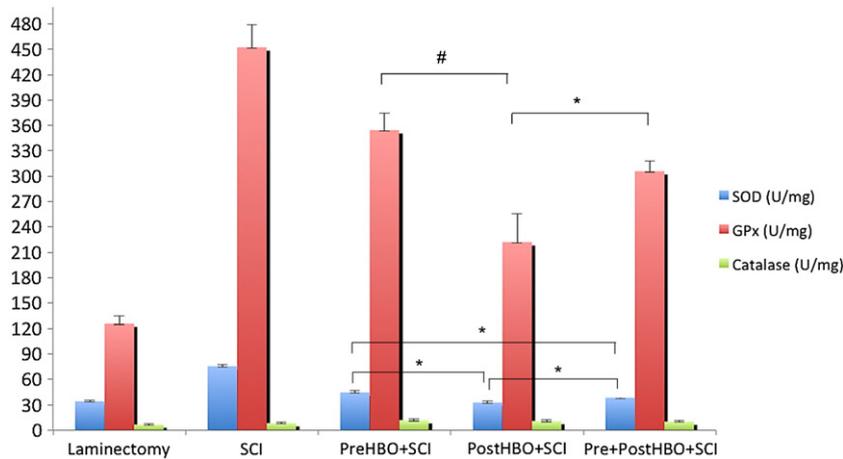
Rats with SCI showed a significant increase ( $p < 0.05$ ) in SOD, GPx, NO<sup>-</sup> and NOS levels when compared to the laminectomy group. In the preopHBO group, a significant decrease was observed in NOS and NO levels when compared to the SCI group ( $p < 0.05$ ). Similarly, there was a significant decrease in SOD, NOS and NO levels in the postopHBO group when compared to the SCI group ( $p < 0.05$ ). In the prepostopHBO group, SOD, GPx, NOS and NO levels decreased when compared to the SCI group ( $p < 0.05$ ). The postopHBO group presented a significant decrease ( $p < 0.05$ ) in SOD levels when compared to the preopHBO group. There was a significant decrease ( $p < 0.05$ ) in SOD levels in the prepostopHBO group when compared to the preopHBO. The prepostopHBO group showed a significant decrease in GPx levels compared to the postopHBO group ( $p < 0.05$ ). There was a significant decrease in GPx levels in the postopHBO group when compared to the preopHBO group (Figs. 1, 2).

### Functional effects of HBO treatment

When compared to the laminectomy group, the SCI group presented significantly lower BBB scores ( $p < 0.05$ ). There was an improvement in both postopHBO and prepostopHBO groups when compared to the SCI group ( $p < 0.05$ ). No significant difference was found in BBB scores between SCI and preopHBO groups and between postopHBO and prepostopHBO groups ( $p > 0.05$ ) (Fig. 3).

## Discussion

HBO treatment was first applied in SCI by Maeda (1965) who suggested tissue ischemia resulted in hypoxia following SCI in dogs, and HBO treatment at 2 ATA provided a dramatic increase in spinal cord tissue oxygen levels. Hartzog et al. (1969) identified a reversal of SCI following treatment at 3 ATA within the first 24 h. Yeo et al. (1976) found that HBO treatment increased motor recovery and decreased spinal degeneration when applied within 2 h following injury and recovery of motor function was observed within the following 8 weeks. A reduction in spinal cord tissue cystic necrosis and damage in the surrounding white matter following HBO treatment



**Fig. 1.** Levels of SOD, GPx and catalase in laminectomy, SCI, PreHBO + SCI (preoperative HBO treatment for 5 days followed by SCI), postHBO + SCI (SCI and postoperative HBO treatment for 7 days) and Pre + PostHBO + SCI (preoperative HBO treatment for 5 days followed by SCI and postoperative HBO treatment for 7 days) groups (# $p < 0.01$ , \* $p < 0.05$ ).

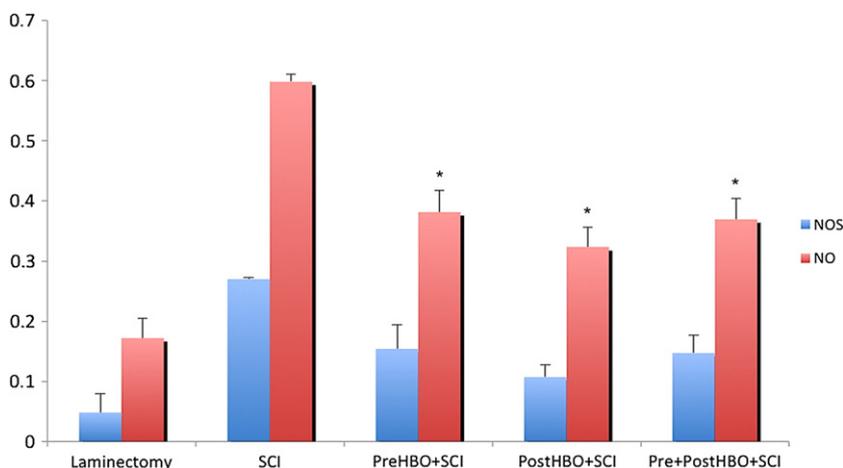
was also observed by [Yeo et al. \(1977\)](#). HBO reduced apoptosis following SCI ([Calvert et al. 2006](#), [Peng et al. 2008](#), [Wang et al. 2009](#)). [Higgins et al. \(1981\)](#) studied spinal cord electropotentials and showed that early HBO treatment contributed to the prevention of progressive degeneration in SCI.

[Neubauer and Walker \(1998\)](#) found that HBO treatment contributed to regeneration in patients after omentum transposition and related cell transplantation procedures following SCI. [Al-Waili et al. \(2005\)](#) reported that HBO application is beneficial in patients with vascular damage in the spinal cord. They suggested that the hemorrhage-reducing effect of HBO treatment leads to a decrease in the amount of environmental iron, which, in turn, reduces free radical formation. [Gelder et al. \(1983\)](#) showed that HBO treatment following SCI led to less cavitation and better vascularized scars containing densely packed collagen fibers. The use of HBO treatment in SCI is not limited to regeneration. [Hart et al. \(1984\)](#) found that HBO treatment led to an increase in the exercise capacity of patients with SCI. Considering the fact that most patients are paraplegic after SCI and they subsequently have rehabilitative needs, they benefit from HBO application.

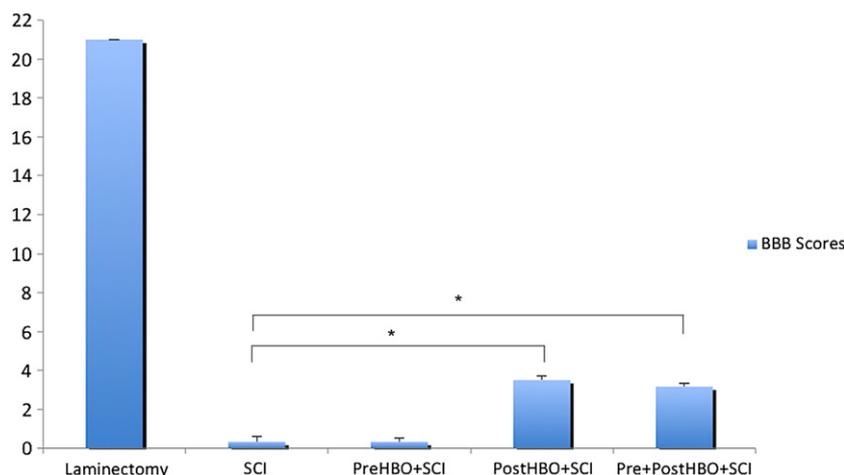
In the present study, post-operative HBO treatment improved neurological recovery significantly, based on BBB scores ( $p < 0.05$ ). There was also a significant increase in SOD, GPx, NOS and NO<sup>-</sup> in

the SCI group when compared to the laminectomy group ( $p < 0.05$ ). However, no significant difference was found for catalase ( $p > 0.05$ ). Tissue destruction secondary to ischemia leads to a rapid increase in SOD levels and destroys the O<sub>2</sub> radical. The emerging O<sub>2</sub> radical must be degraded to H<sub>2</sub>O<sub>2</sub> by SOD enzyme. If this degradation does not occur fast enough, it converts into OH<sup>-</sup>, the most dangerous free radical in neural tissue, or the O<sub>2</sub> radical reacts with nitrogen and leads to free nitrogen radicals (NOO<sup>-</sup>) formation. Then, the emerging H<sub>2</sub>O<sub>2</sub> is degraded to H<sub>2</sub>O with GPx or catalase, which utilizes the same substrate. Similar to SOD values, we also found a significant increase in GPx levels, which indicates that the injury induced free radical formation. Also, there was a more significant decrease in NO<sup>-</sup> and NOS levels in the respective group when compared to controls. Catalase utilizes H<sub>2</sub>O<sub>2</sub> as does GPx, however, it requires a higher substrate concentration. In this study, the significant increase in GPx and the lack of such an increase in catalase were attributed to the low substrate concentration.

[Higgins et al. \(1981\)](#) suggested that HBO treatment preserved the seriously injured neurons of the long tracts of the spinal cord during the early stages of traumatic SCI. HBO treatment increased arterial and cerebrospinal fluid PO<sub>2</sub> ([Holbach et al. 1977](#)). In the present study, when compared to the only-SCI group, the SCI group that received HBO



**Fig. 2.** Levels of NOS and NO in laminectomy, SCI, PreHBO + SCI (preoperative HBO treatment for 5 days followed by SCI), postHBO + SCI (SCI and postoperative HBO treatment for 7 days) and Pre + PostHBO + SCI (preoperative HBO treatment for 5 days followed by SCI and postoperative HBO treatment for 7 days) groups (\* $p < 0.05$ ).



**Fig. 3.** BBB scores in laminectomy, SCI, PreHBO + SCI (preoperative HBO treatment for 5 days followed by SCI), postHBO + SCI (SCI and postoperative HBO treatment for 7 days) and Pre + PostHBO + SCI (preoperative HBO treatment for 5 days followed by SCI and postoperative HBO treatment for 7 days) groups ( $*p < 0.05$ ).

treatment for 7 days postoperatively presented a significant decrease in SOD, NOS and NO<sup>-</sup> levels ( $p < 0.05$ ). This indicates that HBO treatment reduces the formation of free oxygen radicals to a significant extent.

There are a number of studies investigating the optimal duration of HBO treatment. We chose the time frame for the treatment protocol as once daily and for 5 days pre-operatively and up to 7 days post-operatively. This time frame was chosen because Schäbitz et al. (2004) found that HBO treatment for 1 h starting from 2 h after stroke onset is neuroprotective in rats and remains effective up to five days, resulting in improved neurological outcome. Also, Hu et al. (2008) found that HBO treatment for 5 days before traumatic brain injury for 1 h protects brain tissue.

Huang et al. (2003) showed that when initiated 30 min after ischemic injury HBO treatment had a protective effect in rabbits. HBO treatment delayed for 6 h following ischemic injury however did not change the prognosis. Huang et al. (2003) reported that single HBO treatment within 3 h after ischemic injury or serial HBO applications up to the sixth hour improved morphological and neurological recovery, while single HBO treatment or serial HBO applications starting from the 24th hour were not effective. Consistently, in this study, we have found that when compared to the only-SCI group, the groups that received HBO before and after SCI showed significant decreases in SOD, GPx, NOS and NO levels ( $p < 0.05$ ). This decrease in enzyme levels, and the significant decrease in GPx levels observed in the group that received HBO pre- and postoperatively, but not in rats that received less HBO, suggested that the effectiveness of HBO treatment increases with the duration of treatment and longer applications were more effective.

We found a significant decrease in SOD levels was found in the SCI group that received HBO postoperatively when compared to the SCI group that received HBO preoperatively ( $p < 0.05$ ). This decrease in SOD levels indicated that postoperative HBO application was more effective than preoperative HBO application. Moreover, preoperative HBO treatment in the SCI group was also more effective than the control group. The effect of HBO increased by orders of magnitude at the postoperative stage and HBO treatment is especially more effective under hypoxic conditions. When the SCI group that received HBO preoperatively was compared to the SCI group that received HBO both pre- and postoperatively, a significant decrease was observed in SOD levels ( $p < 0.05$ ). This indicates that HBO treatment applied after the injury and in multiple sessions is more effective. When the SCI group that received HBO preoperatively was compared with the SCI group that received HBO both pre- and postoperatively, a significant decrease ( $p < 0.05$ ) was identified in GPx levels in both the pre- and postoperative HBO group. The inverse proportion between the

decrease of GPx levels and the increase in the duration of HBO treatment indicates that HBO mediates the elimination of O<sub>2</sub> radicals in a different manner than H<sub>2</sub>O<sub>2</sub> conversion. The lack of any significant differences in other parameters indicates that HBO is beneficial, but not effective for all parameters. When the SCI group was compared with the group that received HBO for 5 days preoperatively, inflicted with SCI and sacrificed the following day, a significant decrease ( $p < 0.05$ ) in NOS and NO levels was found in the HBO group. This indicates that preoperative HBO treatment is beneficial for nervous tissue regeneration, and NOS and NO are the first parameters to change in the early period of neural injury. However, we did not observe any positive effects of pre-operative HBO treatment on locomotor behavior, indicating that although pre-operative HBO treatment before SCI may be beneficial, it is not effective enough to show any behavioral improvement. Therefore, although some enzyme activities can be altered by pre-injury HBO may in the future lead to insights to mechanism, it is incongruous to discuss pre-injury HBO as a 'treatment' in the context of SCI.

Kahraman et al. (2007) found that HBO administration following SCI diminished SOD and GPx. They also found that HBO induced ROS formation, which was not observed in our study. This may be because they have made the HBO treatment at a higher pressure (4 ATA).

Topuz et al. (2010) combined HBO treatment with hypothermia in rats with SCI and found a significant decrease in SOD, GPx and catalase levels in the spinal cord tissue. We did not observe any difference for catalase, presumably because GPx primarily degrades H<sub>2</sub>O<sub>2</sub> due to the low affinity of catalase enzyme to H<sub>2</sub>O<sub>2</sub>. Consistent with this study, Yu et al. (2004) found a reduction in the number of iNOS immunoreactive neurons at 1 to 2 days after HBO treatment in rats with SCI. Single HBO treatment produced a greater decrease than the once a day HBO treatment at days two to four, suggesting early HBO treatment effectively suppressed the progression of apoptosis perhaps via the inhibition of iNOS.

## Conclusion

HBO is found to be beneficial for neurological recovery when applied before and after SCI. The rats that received HBO before and after SCI showed significant decreases in SOD, GPx, NOS and NO levels. However, no significant difference was found among experimental groups for catalase, indicating that the effect of HBO on catalase levels may not be as beneficiary in SCI treatment; however, further studies are needed to confirm this. Although the present study confirms the efficacy of HBO in SCI in terms of neurological

and biochemical outcomes, the optimal number of applications needs to be further investigated so that the effect of the treatment remains constant.

#### Conflict of interest statement

None.

#### Acknowledgements

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