

# Different dose-dependent effects of ebselen in sciatic nerve ischemia-reperfusion injury in rats

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## ABSTRACT

Ebselen is an organoselenium compound which has strong antioxidant and anti-inflammatory effects. We investigated the neuroprotective role of ebselen pretreatment in rats with experimental sciatic nerve ischemia-reperfusion (I/R) injury. Adult male Sprague Dawley rats were divided into four groups (N = 7 in each group). Before sciatic nerve I/R was induced, ebselen was injected intraperitoneally at doses of 15 and 30 mg/kg. After a 2 h ischemia and a 3 h reperfusion period, sciatic nerve tissues were excised. Tissue levels of malondialdehyde (MDA) and nitric oxide (NO), and activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) were measured. Sciatic nerve tissues were also examined histopathologically. The 15 mg/kg dose of ebselen reduced sciatic nerve damage and apoptosis ( $P < 0.01$ ), levels of MDA, NO, and inducible nitric oxide synthase (iNOS) positive cells ( $P < 0.01$ ,  $P < 0.05$ , respectively), and increased SOD, GPx, and CAT activities ( $P < 0.001$ ,  $P < 0.01$ ,  $P < 0.05$ , respectively) compared with the I/R group that did not receive ebselen. Conversely, the 30 mg/kg dose of ebselen increased sciatic nerve damage, apoptosis, iNOS positive cells ( $P < 0.01$ ,  $P < 0.05$ ,  $P < 0.001$ ) and MDA and NO levels ( $P < 0.05$ ,  $P < 0.01$ ) and decreased SOD, GPx, and CAT activities ( $P < 0.05$ ) compared with the sham group. The results of this study suggest that ebselen may cause different effects depending on the dose employed. Ebselen may be protective against sciatic nerve I/R injury via antioxidant and antiapoptotic activities at a 15 mg/kg dose, conversely higher doses may cause detrimental effects.

KEY WORDS: Ebselen; ischemia-reperfusion injury; oxidative stress; sciatic nerve

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## INTRODUCTION

Ischemia plays a major role in the development of pathological changes in many neuropathies. Diabetes mellitus, vascular occlusive diseases, and trauma are some pathologic conditions which cause neuropathy associated with ischemia of peripheral nerves [1]. A nerve must have a continuous and sufficient oxygen supply through intrinsic and extrinsic microvascular systems to carry out its normal functions [2]. Ischemia and reperfusion (I/R) causes ischemic fiber degeneration and oxidative injury in peripheral nerves. Schwann cells are the main target of oxidative injury due to I/R [3]. The

neural tissue damage from I/R has ranged from demyelination to axonal degeneration and nerve infarction [4]. The degree of neural tissue damage is related to the duration of ischemia; the cellular and biochemical interactions which occur as a result of reperfusion may exacerbate tissue damage [5].

Ebselen [2-phenyl-1,2-benziselenazol-3(2H)-one; PZ-51; DR-3305] is a synthetic anti-inflammatory, antioxidant, selenium-organic compound that mimics glutathione peroxidase (GPx) activity by catalyzing the reduction of reactive oxygen species (ROS) in a manner similar to GPx. It reacts with peroxynitrites generated during I/R and reduces hydroperoxides, including those arising in biomembranes and lipoproteins, to the corresponding hydroxy compounds. In addition, it reacts with a number of thiols such as glutathione, N-acetyl-L-cysteine, and dithiothreitol. At low concentrations, ebselen also inhibits a number of enzymes involved in inflammation, such as lipoxygenases, nitric oxide synthases, nicotinamide adenine

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dinucleotide phosphate (NADPH) oxidase, protein kinase C, and H<sup>+</sup>/K<sup>+</sup>-ATPase. This inhibitory effect may contribute to the anti-inflammatory potential of ebselen [6,7]. Ebselen is a lipid-soluble compound, therefore, it readily enters the cell. It exhibits strong electrophilic activity, which makes it capable of forming selenenyl-sulfide bonds with cysteine residues in proteins. The main mechanism of action of ebselen is through reactions with cysteine thiol groups in proteins. This compound also reacts with the thioredoxin (Trx) system and it is a highly suitable substrate for the mammalian thioredoxin reductase (TrxR) and acts as a highly efficient oxidant of the reduced Trx [7].

In this study, we investigated the effectiveness of ebselen in protecting the sciatic nerve against I/R injury, using biochemical (malondialdehyde, MDA; nitric oxide, NO; superoxide dismutase, SOD; glutathione peroxidase, GPx; and catalase, CAT), immunohistochemical (terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end-labeling, TUNEL; inducible nitric oxide synthase, iNOS; and proliferating cell nuclear antigen, PCNA), and histopathological analyses. Furthermore, we compared the protective effects of two different doses of ebselen, namely 15 mg/kg and 30 mg/kg, to determine an appropriate dosage for preventative treatment to minimize sciatic nerve I/R injury. To the best of our knowledge, the effect of ebselen has not been previously studied in rats with the sciatic nerve I/R injury using these biochemical and immunohistochemical parameters.

## MATERIALS AND METHODS

This study was carried out in the Experimental Animals Research Center of Dumlupinar University. The study experiments were approved by the Local Ethics Committee for Experiments on Animals of Dumlupinar University (No: 2014.06.01). All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animal Resources Commission on Life Sciences National Research Council [8].

### Animals

Male Sprague-Dawley rats (300–350 g, 12–16 weeks, N = 28) were used. All rats were housed individually in transparent polycarbonate cages with a 12:12 h light-dark cycle at 22 ± 2 °C and provided ad libitum access to fresh water and standard rat chow until the experiments commenced.

### Chemicals

Ebselen [2-phenyl-1,2-benziselenazol-3(2H)-one] was purchased from Sigma-Aldrich Co. LLC (Ebselen<sup>®</sup>, St. Louis, MO, USA). It was dissolved in 10% Tween 80 (Sigma–Aldrich Co. LLC., St. Louis, MO, USA) [9].

## Experimental study design

Experimental animals were randomly divided into four groups (N = 7). Group I, (sham) were subjected to abdominal median laparotomy and not to I/R conditions. Rats were treated with intraperitoneal (i.p.) injections of 1 mL of vehicle (10% Tween 80) 24 h before the laparotomy. Group II (I/R) was subjected to 2 h of ischemia followed by 3 h of reperfusion. The rats were treated with i.p. injections of 1 mL of vehicle 24 h before the laparotomy. Group III (I/R with 15 mg/kg ebselen) was treated similarly to the I/R group, but also received i.p. injections of 1 mL of 15 mg/kg ebselen, 24 h before the I/R. Group IV (I/R with 30 mg/kg ebselen) was treated similarly to Group III rats but received 1 mL of 30 mg/kg ebselen [10].

## Surgical procedures

Rats were weighed and then anesthetized with intramuscular (i.m.) injections of 10 mg/kg xylazine hydrochloride (Rompun, Bayer, Istanbul, Turkey) and 70 mg/kg ketamine (Ketalar<sup>®</sup>, Pfizer, Istanbul, Turkey). After a suitable level of anesthesia was achieved, the rats were placed on a homeothermic table to maintain a stable body temperature of 37 ± 1 °C, and then the anterior abdominal wall was shaved and sterilized with povidone-iodine solution. Ischemia was produced by ligation of the abdominal aorta at the level of iliolumbar arteries; these arteries supply the hindlimbs. The aorta and its branches were occluded with an atraumatic vascular clamp (Vascu<sup>®</sup>Stop Bulldog Clamp) for 2 h [11]. After 2 h of ischemia, the clamps were removed to allow reperfusion for 3 h. The abdomen was subsequently closed and the rats were observed during the 3 h of reperfusion. After reperfusion, the animals were euthanized under anesthesia, and the sciatic nerves were excised bilaterally. The sciatic nerve tissue samples were rinsed with a cold heparinized phosphate-buffered saline (PBS) to remove any red blood cells or clots. A portion of the sciatic nerve samples were fixed with 10% buffered formalin for histopathological and immunohistochemical analyses. Additional portions of sciatic nerve tissue samples were placed in Eppendorf tubes and immediately stored at -80°C for biochemical analysis.

## Biochemical analysis

For biochemical analysis, nerve tissue was mixed with cold working solution (50 mM phosphate buffer, pH 7.40), and homogenized with a mechanic homogenizer (Analytik Jena speedmill plus, Germany). The mixture was then centrifuged at 10,000 × g for 15 min at 4°C, and the supernatant was preserved for biochemical analysis by storing on ice. MDA and NO levels, as well as SOD, GPx, and CAT activities were measured in sciatic nerve tissue homogenates using commercial

enzyme-linked immunosorbent assay (ELISA) kits (Cayman Inc, Ann Arbor, MI, USA) on a microplate reader (BMG Labtech Spectrostar Nano, GmbH, Ortenberg, Germany). MDA concentration was measured based on the thiobarbituric acid reactive substances (TBARS) method [12]. NO concentration was measured based on the Griess method [13]. SOD activity was measured using a salt of tetrazolium to detect superoxide radicals generated by xanthine oxidase and hypoxanthine, in the presence of a range of concentrations of SOD [14]. Measurement of GPx activity was based on the method of Paglia and Valentine [15]. CAT activity was measured based on the reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide. The formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (purpald) as a chromogen [16]. Tissue protein concentrations were measured based on the Bradford method on a Beckman Coulter AU680 analyzer (Beckman Coulter, Miami, FL, USA) [17].

### Histopathological examinations

Sciatic nerve tissue samples were fixed in 10% formalin, embedded in paraffin, sectioned (4  $\mu\text{m}$ ), placed on slides, stained with hematoxylin and eosin (H&E), and examined under a light microscope (Olympus BX51, Tokyo, Japan) by a pathologist who was blinded to the treatment groups. The pathologic slides were graded histopathologically according to the edema and axonal vacuolization using a previously described scoring system by Coban et al. [18]. For each section, the vacuolization and edema were semi-quantitatively graded from 0 to 3 as follows: 0-normal, 1-mild, 2-moderate, and 3-severe.

### *In situ* detection of sciatic nerve apoptosis

We used an *in situ* TUNEL assay to assess the degree of sciatic nerve apoptosis. Formalin-fixed sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to water. DNA fragmentation in apoptosis was detected using a commercially available kit (ApopTag® peroxidase *in situ* apoptosis detection kit, Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Processed samples were examined under a light microscope (Olympus BX51, Tokyo, Japan). The number of TUNEL-positive cells per 100 was calculated from randomly selected fields. The apoptotic index was calculated as the percentage of apoptotic (TUNEL-positive stained) cells.

### Immunohistochemical examinations

iNOS and PCNA were examined immunohistochemically. Formaldehyde-fixed tissue was embedded in

paraffin for further immunohistochemical examination. Immunohistochemical staining was performed using commercially available kits (Thermoscientific Lab Vision, Fremont, CA, USA) according to the manufacturer's instructions and examined under a light microscope (Olympus BX51). The percentage of immunopositive cells was determined.

### Statistical analyses

Statistical analyses were performed using GraphPad Prism version 6.05 (GraphPad Software, Inc., CA, USA). All data were expressed as mean  $\pm$  standard error of the mean (SEM). Because of the small experimental groups, we used non-parametric statistics. Quantitative data were tested using the Kruskal-Wallis analysis of variance on ranks and Dunn's method was used for *post hoc* testing. A  $P$  value  $< 0.05$  was considered to be statistically significant.

## RESULTS

Significant differences were observed between MDA and NO levels in the assay groups ( $P < 0.001$ ). Our results revealed that tissue MDA and NO levels were significantly higher in the I/R group compared to the sham group ( $P < 0.001$ , Table 1). Tissue MDA and NO levels were also significantly higher in the I/R with 30 mg/kg ebselen group compared with the sham group ( $P < 0.05$  and  $P < 0.01$ , respectively, Table 1). When the I/R with 15 mg/kg ebselen and the I/R groups were compared, MDA and NO levels showed significant differences ( $P < 0.01$  and  $P < 0.05$ , respectively, Table 1).

When SOD, GPx, and CAT activities were evaluated, significant differences were observed between assay groups ( $P < 0.001$ , Table 1). SOD, GPx, and CAT activities were significantly lower in the I/R group compared to the sham group ( $P < 0.001$ , Table 1). Significant differences were observed between the I/R with 15 mg/kg ebselen and the I/R groups for SOD, GPx, and CAT ( $P < 0.01$ ,  $P < 0.01$ ,  $P < 0.05$ , respectively, Table 1). SOD, GPx, and CAT activities were significantly lower in the I/R with 30 mg/kg ebselen group compared to sham group ( $P < 0.05$ , Table 1).

In the histopathological examinations, our results showed that I/R injury caused a marked increase in total histopathological sciatic nerve damage score compared with the sham group ( $P < 0.001$ , Table 2). The dose of 15 mg/kg of ebselen significantly reduced the histological damage scores compared with the I/R group ( $P < 0.01$ , Table 2). Although the 15 mg/kg dose of ebselen significantly attenuated morphologic changes, which occurred as a result of I/R injury in the sciatic nerve tissue, the 30 mg/kg dose of ebselen did not reduce the histological damage scores compared to the I/R group ( $P > 0.05$ , Table 2). Furthermore, the 30 mg/kg dose of

**TABLE 1.** Comparisons of measured biochemical parameters in sciatic nerve tissue samples between assay groups

Biochemical parameters (mean±SEM)	GI (Sham) (N=7)	GII (I/R) (N=7)	GIII (I/R with 15 mg/kg Ebselen) (N=7)	GIV (I/R with 30 mg/kg Ebselen) (N=7)	Statistical analysis (P values)
MDA (µmol/g protein)	8.67±0.29	20.66±0.93 <sup>a</sup>	10.58±0.57 <sup>d</sup>	14.36±0.59 <sup>b</sup>	<i>P</i> <0.001
NO (µmol/g protein)	0.20±0.01	1.26±0.18 <sup>a</sup>	0.53±0.04 <sup>e</sup>	0.81±0.05 <sup>c</sup>	<i>P</i> <0.001
SOD (U/mg protein)	15.52±0.99	4.01±0.42 <sup>a</sup>	12.38±0.78 <sup>d</sup>	7.80±0.94 <sup>b</sup>	<i>P</i> <0.001
GPx (nmol/min/mg protein)	131.7±5.28	26.27±2.29 <sup>a</sup>	95.03±4.08 <sup>d</sup>	69.61±3.78 <sup>b</sup>	<i>P</i> <0.001
CAT (nmol/min/mg protein)	14.50±0.49	3.06±0.24 <sup>a</sup>	8.09±0.63 <sup>c</sup>	5.51±0.66 <sup>b</sup>	<i>P</i> <0.001

MDA: Malondialdehyde, SOD: Superoxide dismutase, NO: Nitric oxide, GPx: Glutathione peroxidase, CAT: Catalase, I/R: Ischemia-reperfusion, SEM: Standard error of the mean. Data are means±S.E.M. of seven rats for each group. <sup>a</sup>*P*<0.001, <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01, compared to sham group; <sup>d</sup>*P*<0.01, <sup>e</sup>*P*<0.05, compared to I/R group. Data were tested using the Kruskal-Wallis Analysis of Variance on Ranks and Dunn's method was used for Post hoc testing. A *P* value of less than 0.05 was considered as statistically significant

**TABLE 2.** Comparisons of scored histopathological and immunohistochemical values in sciatic nerve tissue samples between assay groups

Scored values (mean±SEM)	GI (Sham) (N=7)	GII (I/R) (N=7)	GIII (I/R with 15 mg/kg Ebselen) (N=7)	GIV (I/R with 30 mg/kg Ebselen) (N=7)	Statistical analysis (P values)
Sciatic nerve damage	0.0±0.0	2.71±0.18 <sup>a</sup>	0.42±0.20 <sup>d</sup>	2.28±0.28 <sup>b</sup>	<i>P</i> <0.001
Apoptosis index (TUNEL)	2.28±0.71	30.0±1.0 <sup>b</sup>	4.14±1.35 <sup>d</sup>	26.43±2.17 <sup>ce</sup>	<i>P</i> <0.001
iNOS	0.0±0.0	3.71±0.18 <sup>a</sup>	1.0±0.0	3.57±0.29 <sup>a</sup>	<i>P</i> <0.001
PCNA	0.0±0.0	22.29±1.98 <sup>b</sup>	16.0±1.49	29.14±4.82 <sup>a</sup>	<i>P</i> <0.001

TUNEL: Terminal deoxynucleotidyl transferase mediated d-UTP nick end-labeling, iNOS: Inducible nitric oxide synthase, PCNA: Proliferating cell nuclear antigen, I/R: Ischemia-reperfusion, SEM: Standard error of the mean. Data are means±S.E.M. of seven rats for each group. <sup>a</sup>*P*<0.001, <sup>b</sup>*P*<0.01, <sup>c</sup>*P*<0.05 compared to sham group; <sup>d</sup>*P*<0.01, compared to I/R group; <sup>e</sup>*P*<0.05, compared to I/R with 15 mg/kg Ebselen group. Data were tested using the Kruskal-Wallis analysis of variance on ranks and Dunn's method was used for Post hoc testing. A *P* value of less than 0.05 was considered as statistically significant. The number of TUNEL-positive cells per 100 was calculated from randomly selected fields. The apoptotic index was calculated as the percentage of apoptotic (TUNEL-positive stained) cells. The percentages of PCNA-positive and iNOS-positive cells were determined

ebselen caused a significant increase in the total histopathological sciatic nerve damage score compared with the sham group (*P* < 0.01, Table 2). Compared with the sham group, a significant increase in axonal cytoplasmic vacuolization and degeneration in axons of the sciatic nerve was observed in the I/R group and I/R with 30 mg/kg ebselen group. In addition, intramyelinic edema within nerve fibers was observed, not only in the perivascular region, but also in endoneurial vessels in the I/R group and I/R with 30 mg/kg ebselen group. However, the histological appearance was near normal in the I/R with 15 mg/kg ebselen group. Considerably preserved neuronal structures were seen in the sciatic nerve pretreated with a 15 mg/kg dose of ebselen (Figure 1A, 1B, 1C, 1D).

When apoptosis in the sciatic nerve tissue was examined using the TUNEL method, we observed that I/R injury caused a significant increase in the apoptotic index compared with the sham group (*P* < 0.01). A dose of 15 mg/kg of ebselen significantly reduced the apoptotic index compared with the I/R group (*P* < 0.01). However, the 30 mg/kg dose of ebselen increased the apoptotic index compared with the sham group and the I/R with 15 mg/kg ebselen group (*P* > 0.05 and *P* < 0.05, respectively, Table 2, Figure 2A, 2B, 2C, 2D).

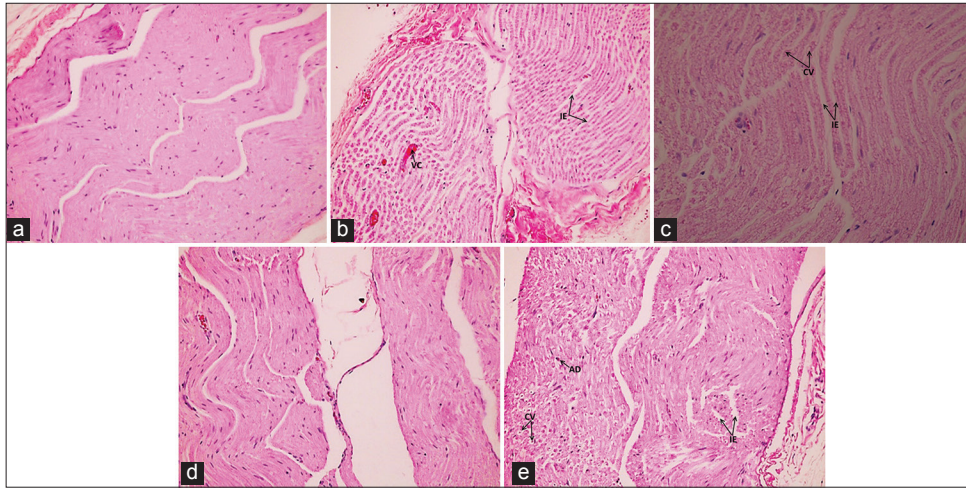
iNOS and PCNA expressions were examined using immunohistochemical staining. Our results demonstrated that I/R injury caused a significant increase in the number of iNOS and PCNA positive cells compared with the sham group (*P* < 0.001 and *P* < 0.01, respectively). The 15 mg/kg dose of ebselen partially reduced the number of iNOS and PCNA

positive cells, but these reductions were not statistically significant (Table 2, Figure 3A, 3B, 3C, 3D and Figure 4A, 4B, 4C, 4D). Moreover, the 30 mg/kg dose of ebselen also significantly increased the number of iNOS and PCNA positive cells compared with sham group (*P* < 0.001, Table 2, Figure 3A, 3B, 3C, 3D and Figure 4A, 4B, 4C, 4D).

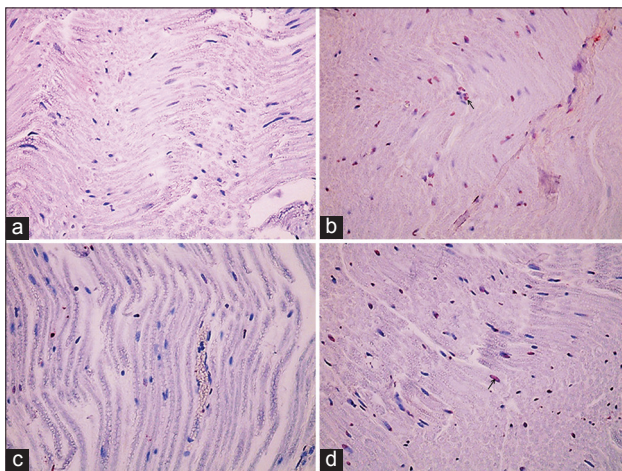
## DISCUSSION

In this study, we investigated the neuroprotective effects of ebselen pre-treatment with two different doses in rats subjected to experimental sciatic nerve I/R injury. Our results revealed that the 15 mg/kg dose of ebselen had remarkable protective effects against sciatic nerve I/R injury. This protective effect was evident as a decrease in MDA and NO levels and an increase in antioxidant enzyme activities in sciatic nerve tissue. In addition, the histopathological examination of sciatic nerve injury, apoptosis, and immunohistochemical markers corroborated this result. Although the 30 mg/kg dose of ebselen pretreatment did not worsen the I/R injury, it revealed comparable level of results with those of the no-drug I/R injury group. The 30 mg/kg dose of ebselen pretreatment led to increases in sciatic nerve damage, apoptosis, and oxidative stress compared with sham group. Therefore, we suggest that ebselen may be protective against sciatic nerve I/R injury at 15 mg/kg dose. Conversely, higher doses may be harmful.

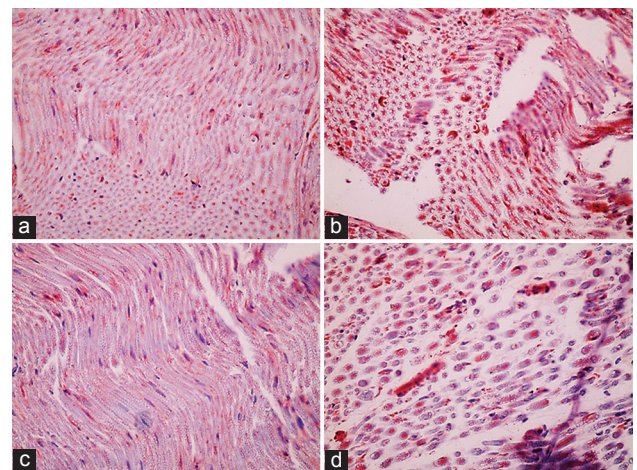
Reperfusion of the ischemic tissue increases the production of ROS, which causes damage to a variety of molecules



**FIGURE 1.** Histopathological examination of the sciatic nerve tissues of assay groups, H&E x 100; H&E x 200. Axonal cytoplasmic vacuolization, degeneration in the axons of the sciatic nerve, intramyelinic edema within nerve fibers in the perivascular region and in endoneurial vessels were seen in the I/R group (B: H&E x 100, B\*: H&E x 200) and I/R with 30 mg/kg ebselen group (D). Considerably preserved neuronal structures were seen in the sciatic nerve treated with a 15 mg/kg dose of ebselen (C). IE: Intramyelinic edema, VC: Vascular congestion, CV: Cytoplasmic vacuolization, AD: Axonal degeneration. A. Sham group, B. I/R group, C. I/R with 15 mg/kg Ebselen group, D. I/R with 30 mg/kg Ebselen group.



**FIGURE 2.** Examination of apoptosis in sciatic nerve tissues of assay groups, TUNEL x 400. Apoptosis in the sciatic nerve tissue was examined using the TUNEL method. The number of TUNEL-positive cells per 100 was calculated from randomly selected fields. The apoptotic index was calculated as the percentage of apoptotic (TUNEL-positive stained) cells. I/R injury caused a significant increase in the apoptotic index (B). A dose of 15 mg/kg of ebselen significantly reduced the apoptotic index (C). The 30 mg/kg dose of ebselen increased the apoptotic index compared with the sham group (D). A. Sham group, B. I/R group, C. I/R with 15 mg/kg ebselen group, D. I/R with 30 mg/kg ebselen group.

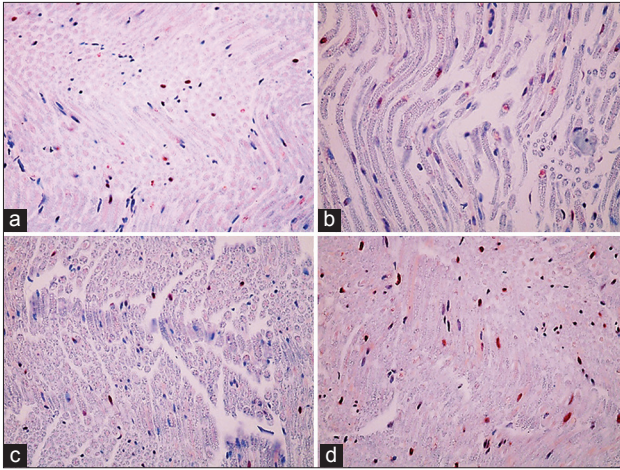


**FIGURE 3.** Examination of iNOS expression in sciatic nerve tissues of assay groups, iNOS x 400. iNOS expression was examined using immunohistochemical staining. The percentage of iNOS-positive cells was determined. I/R injury caused a significant increase in the number of iNOS positive cells (B). The 15 mg/kg dose of ebselen partially reduced the number of iNOS positive cells (C). The 30 mg/kg dose of ebselen increased the number of iNOS positive cells compared with the sham group (D). A. Sham group, B. I/R group, C. I/R with 15 mg/kg Ebselen group, D. I/R with 30 mg/kg Ebselen group.

and impairs cellular functions [19]. Production and accumulation of ROS causes lipid peroxidation (LPO) in cellular membranes and subsequent generation of MDA. Therefore, MDA is a useful quantitative indicator of LPO [20]. In this study, sciatic nerve I/R caused significant LPO accumulation. Although the 15 mg/kg dose of ebselen significantly reduced LPO, the 30 mg/kg dose of ebselen did not reduce LPO compared with the I/R group. Moreover, the 30 mg/kg dose of ebselen significantly increased LPO accumulation compared with the sham group. The results of this study were consistent with previous

studies [21-23]. Consequently, while the 15 mg/kg dose of ebselen pretreatment may be protective against sciatic nerve I/R injury by reducing LPO, the 30 mg/kg dose of ebselen appears to have little or no effect on LPO.

Nitric oxide is a signaling molecule produced in various cell types in the nerve tissue, and is synthesized by two isoforms of NOS: neuronal and endothelial. A third isoform of NOS, namely iNOS, is expressed in numerous cell types as a result of the inflammatory processes. Higher levels of NO, which are produced by iNOS, contribute to the inflammatory process



**FIGURE 4.** Examination of PCNA expression in sciatic nerve tissues of assay groups, PCNA x 400. PCNA expression was examined using immunohistochemical staining. The percentage of PCNA positive cells was determined. I/R injury caused a significant increase in the number of PCNA positive cells (B). The 15 mg/kg dose of ebselen partially reduced the number of PCNA positive cells (C). The 30 mg/kg dose of ebselen increased the number of PCNA positive cells compared with the sham group (D). A. Sham group, B. I/R group, C. I/R with 15 mg/kg Ebselen group, D. I/R with 30 mg/kg Ebselen group.

and induce I/R injury [24-26]. Our results showed that the 15 mg/kg dose of ebselen reduced the tissue NO levels and the number of iNOS positive cells compared with the I/R group. However, the 30 mg/kg dose of ebselen increased tissue NO levels and the number of iNOS positive cells compared with the sham group. Our results were consistent with a previous study [27]. Unlike our study, Wei *et al.* demonstrated that both a 10 mg/kg and a 30 mg/kg dose of ebselen administered by an intragastric route significantly reduced the NO and iNOS mRNA levels in rats with a traumatic brain injury model in a dose-dependent manner [28]. In our study, a 30 mg/kg dose of ebselen did not reduce NO levels or the number of iNOS positive cells. This discrepancy may be related to differences in bio-availability and the LD<sub>50</sub> dose of ebselen between an oral and a parenteral route. In addition, the dose-dependent effect of ebselen may be different in brain and sciatic nerve tissues. The results of this study led us to propose that the 15 mg/kg dose of ebselen may be neuroprotective against I/R injury in sciatic nerve by decreasing NO levels and inhibiting iNOS activity. However, the 30 mg/kg dose of ebselen appears to have little or no effect on iNOS activity.

Endogenous antioxidant enzymes are an important defense system that scavenges ROS including SOD, GPx, and CAT, thereby reducing their detrimental effects [29]. In this study, the 15 mg/kg dose of ebselen significantly increased antioxidant enzyme activities. However, the 30 mg/kg dose of ebselen did not significantly increase antioxidant enzyme activities. Our results were consistent with previous studies [21-23,30,31]. Therefore, we suggest that the

protective effects of the 15 mg/kg dose of ebselen may be exerted via improvement of the antioxidant defense system in sciatic nerve I/R injury.

Apoptosis triggered by oxidative stress is an important pathogenic mechanism in I/R-induced cell death [32]. In this study, results of the TUNEL assay demonstrated that the 15 mg/kg dose of ebselen reduced the apoptotic index. Conversely, the 30 mg/kg dose of ebselen significantly increased the apoptotic index. Our results were consistent with previous studies [30,33-36]. Yang *et al.* demonstrated that ebselen led to a dose-dependent increase of TUNEL-positive cells, which depleted intracellular glutathione (GSH) and protein thiols [35]. The authors have suggested that the rapid depletion of intracellular thiols and GSH may lead to a severe imbalance of the cellular redox status, and that this depletion may play a role in ebselen-induced apoptosis [35]. Another study reported that, although a low dose of ebselen revealed a protective effect against glutamate toxicity by decreasing Bax and increasing Bcl-2, higher doses caused a significant decrease both in cell viability and protection against glutamate toxicity. This suggests that the ebselen lacks neurotrophic effects and, at higher concentrations, is toxic to cells [36]. Consequently, our results indicate that the 15 mg/kg dose of ebselen may ameliorate sciatic nerve I/R injury by inhibiting the apoptotic pathways. Conversely, the 30 mg/kg dose of ebselen may induce apoptosis by activating the apoptotic pathways.

Paunesku *et al.* used PCNA immunostaining to detect proliferating cells. PCNA is a nuclear auxiliary protein of DNA polymerase  $\delta$  which accumulates in the nucleus during the S phase of the cell cycle; it is required for DNA replication and repair. Because its expression correlates to cell proliferation and cell cycle, it is commonly used as a physiological or pathological marker of proliferating cells [37]. Schwann cells of normal peripheral nerves were reported to be immunoreactive for PCNA [38]. In addition, it has been reported that PCNA mRNA is expressed not only in proliferating cells but also in nonproliferating cells such as neurons [39]. Deng *et al.* demonstrated that the proliferation of Schwann cells and PCNA increased after sciatic nerve injury. The researchers suggested that the proliferation of Schwann cells is important for peripheral nerve regeneration and that they proliferate during myelination after sciatic nerve injury [40]. In our study, the no-drug I/R group and the group given 30 mg/kg ebselen both significantly increased the number of PCNA positive cells compared with the sham group. Although the PCNA positivity was increased in the I/R with 15 mg/kg ebselen group, this increase was not significant compared with the sham group. Consequently, we suggest that because the sciatic nerve tissue was well protected by the dose of 15 mg/kg ebselen against I/R injury, the increase in PCNA and the corresponding proliferation of Schwann cells may be less in the

I/R with 15 mg/kg ebselen group than in the I/R and the I/R with 30 mg/kg ebselen groups because of a reduced requirement for regenerating tissue.

The most interesting result of this study was the dose-dependent manner of the effect of ebselen in relation to sciatic nerve I/R injury. In spite of the positive effects of ebselen, certain signs were observed in response to selenium-containing compounds that could be the basis of potentially damaging actions. Several *in vitro* studies reported that high concentrations of ebselen may be toxic to cells [35,41-43]. Although the mechanism underlying the toxicity of ebselen is not fully understood, it has been shown that ebselen treatment causes a dose-dependent depletion of cellular GSH levels [35,41]; a dose-dependent loss of mitochondrial membrane potential and release of cytochrome c [42]; and deterioration of mitochondrial functioning and inhibition of the Trx system [43]. Consequently, in our study, while the 15 mg/kg dose of ebselen demonstrated a protective effect against sciatic nerve I/R injury, the 30 mg/kg dose of ebselen did not demonstrate any protective effect. Ebselen may be neuroprotective at low concentrations, however, it may cause detrimental effects at high concentrations.

In conclusion, the results of this study revealed that the low and high doses of ebselen have different effects in sciatic nerve I/R injury. Ebselen may be an effective neuroprotective agent at low doses against sciatic nerve I/R injury depending on antioxidant and antiapoptotic effects. On the other hand, high doses of ebselen may cause detrimental effects, therefore, these observations are of particular importance because ebselen is currently used in clinical trials for possible use as a therapeutic agent for stroke. Further studies should be performed to identify a reliable therapeutic window for this promising, clinically used agent.

## DECLARATION OF INTERESTS

The authors declare that they have no conflicts of interest to disclose.

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