Abstract. Ebselen is an organoselenium compound that has demonstrated potent antioxidant and anti-inflammatory effects in previous studies. The present study was conducted to evaluate the effect of ebselen on myocardial ischemia-reperfusion (I/R) injury in a rat model and to elucidate the related mechanisms. Myocardial infarct size was assessed using triphenyltetrazolium chloride staining. Myocardial injury was evaluated according to the histopathological and ultrastructural alterations of rat hearts and the serum activity levels of cardiac enzymes, including creatine kinase (CK), CK-MB isoenzyme and lactate dehydrogenase (LDH). Cardiomyocyte apoptosis was detected using the terminal dUTP nick end-labelling (TUNEL) assay. In addition, the expression of apoptosis-associated proteins was measured using western blot analysis. In heart tissue specimens the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx), and levels of malondialdehyde (MDA) and protein carbonyl (PC) were also detected. The results indicated that ebselen reduced I/R-induced increase in myocardial infarct size and prevented the I/R-induced decreases in ejection fraction and fractional shortening. Further of note, ebselen improved I/R-induced rat heart injury. This was indicated by attenuation of histological and ultrastructural changes; reduction of serum CK, CK-MB and LDH activity levels; and decreased cell apoptosis on TUNEL staining, which was verified by decreased expression of cleaved (C)-Caspase-8, C-Caspase-3, B-cell lymphoma 2 (Bcl-2)-associated X protein and C-PARP, and increased expression of Bcl-2. Additionally, SOD and GPx activity levels were significantly higher, while MDA and PC levels were significantly lower in the ebselen + I/R group compared with in the I/R group. In conclusion, the present results suggested that ebselen serves an important role in protecting against myocardial I/R injury. The underlying mechanism may involve suppression of cardiomyocyte apoptosis and promotion of antioxidant activity.

Introduction

Interruption of blood supply to tissues results in ischemic injury. However, restoration of the blood supply initiates a chain of events that may result in additional cell injury, which is known as reperfusion injury (1,2). Myocardial ischemia-reperfusion (I/R) injury has crucial roles in inducing cardiomyocyte apoptosis, necrosis and left ventricular remodeling, which can result in irreversible consequences, including myocardial infarction, progressive deterioration of cardiac function and heart failure (3,4). In myocardial I/R injury, redox imbalances trigger a number of signaling pathways mediated by free radicals including reactive nitrogen species (RNS) and reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals, hydrogen peroxide, nitric oxide and peroxynitrite (5-7). In myocardial ischemia, hypoxia and reoxygenation, which may induce the excessive production of RNS and ROS in cardiac tissues, are principal causes of reperfusion injury (8). Free radicals are typically scavenged by antioxidant enzymes, including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), which serve important roles in the prevention of lipid peroxidation (9,10). Furthermore, previous studies have revealed that the antioxidant activities of these enzymes may be reduced in patients following myocardial infarction or in those with ischemic heart disease (11,12).
Previous studies have reported some effective strategies for protecting the heart against I/R injury in rat models; however, these strategies have been mostly unsatisfactory in limiting oxidative damage in clinical trials (13-15). Ebselen [2-phenyl-1, 2-benzisothiazol-3(2H)-one] is a selenium-containing organic compound that is described as a GPx mimic and has protective effects against oxidative injury (16,17). Since oxygen free radical scavengers serve an important role in the attenuation of I/R injury, the present study evaluated the effects of ebselen, a potent scavenger of peroxynitrite, against myocardial I/R injury in a rat model.

Materials and methods

Animals and myocardial I/R model. A total of 32 male Sprague-Dawley rats (260-300 g, 3-4 weeks old) were obtained from the Laboratory Animal Center of Wuhan University, Wuhan, China. The rats were maintained with standard feeding conditions including adequate food and water ad libitum, a 12 h light/dark, 20-25°C and 50-65% humidity. Following a 12-h fasting period, all rats were anesthetized with 10% ketamine (80 mg/kg; Jiangsu Hengrui Pharmaceutical Co., Ltd., Lianyangung, China) and 2% xylazine (5 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) by intraperitoneal injection. Subsequently, surgery was performed on rats at room temperature (22-24°C). Following skin incision, the hearts were exposed through a left thoracotomy in the fourth intercostal space. A 6-0 silk suture was placed 1-2 cm from the root of the left anterior descending coronary artery. The suture was loosened following occlusion for 30 min, which was followed by 2 h of reperfusion. The same surgical procedures without ligation were performed on rats in a sham group. Following this, the skin was sutured, anesthesia was discontinued and the rats were put into pre-warmed cages for recovery. Rats were sacrificed by cervical dislocation under anesthesia with 5.0% isoflurane and placed supinely on a heating pad, which maintained rat body temperature at 36-37°C. Anesthesia was maintained by inhalation of 2% isoflurane, which was driven by 100% oxygen flow and ventilated using a small animal respirator. Two-dimensional M-mode echocardiograms and pulsed-wave Doppler spectral tracings were obtained using a Vivid 7 echocardiography machine (General Electric Healthcare Corporation, Waukesha, WI, USA) equipped with a 10 MHz-phased array transducer. Following this, diastolic left ventricular posterior wall thickness, systolic left ventricular posterior wall thickness, left ventricle diastolic dimension and left ventricle systolic dimension were measured using M-mode tracings. In addition, ejection fraction (EF%) and percent fractional shortening (%FS) were calculated.

Echocardiography. Echocardiography was performed in rats for assessment of cardiac function prior to and following myocardial I/R injury. Rats were anesthetized with 5.0% isoflurane and placed supinely on a heating pad, which maintained rat body temperature at 36-37°C. Anesthesia was maintained by inhalation of 2% isoflurane, which was driven by 100% oxygen flow and ventilated using a small animal respirator. Two-dimensional M-mode echocardiograms and pulsed-wave Doppler spectral tracings were obtained using a Vivid 7 echocardiography machine (General Electric Healthcare Corporation, Waukesha, WI, USA) equipped with a 10 MHz-phased array transducer. Following this, diastolic left ventricular posterior wall thickness, systolic left ventricular posterior wall thickness, left ventricle diastolic dimension and left ventricle systolic dimension were measured using M-mode tracings. In addition, ejection fraction (EF%) and percent fractional shortening (%FS) were calculated.

Histopathological examination of myocardium. Heart tissue samples were obtained and preserved in 2% glutaraldehyde solution for histopathological examination at the end of myocardial reperfusion. For this, heart tissue specimens were transferred into 70% ethanol, embedded in paraffin, sectioned into 5 μm-thick sections, stained with hematoxylin for 5 min and eosin for 3 min at room temperature, and examined by light microscopy.

Electron microscopy analysis. Heart tissue samples were obtained at the end of reperfusion and immediately preserved in 2% glutaraldehyde solution. Samples were fixed in 1% osmic acid for 2-3 h at 4°C, embedded in Epon 812 epoxy resin overnight at 37°C and cut into 5 μm-thick sections. Sections were prepared with acetate double oxygenic uranium for 30 min and citrate lead stain for 10 min at room temperature. Following this, the sections were examined using a JEM-1230 transmission electron microscope (BD Biosciences, Tokyo, Japan).

Terminal dUTP nick end-labelling (TUNEL) assay. The TUNEL assay was used to examine myocardial apoptosis. An in situ cell death detection kit (Roche Diagnostics, Indianapolis, IN, USA) was used according to the manufacturer's instructions. The excised heart tissues were fixed for 24 h using 10% formal acid solution at room temperature and then cut horizontally into 5 μm-thick sections. After washing with phosphate-buffered saline (PBS) twice, the tissue sections were preprocessed with 0.1% Triton X-100 and 0.1% sodium citrate (freshly prepared) for 15 min. Following this, the sections were incubated for 1 h at 37°C with the commercially prepared labelling mixture and stained with...
4,6-diamidino-2-phenylindole for 5 min at room temperature. Three slides from each block were evaluated under a fluorescence microscope to determine the percentage of apoptotic cells. In addition, four fields on each slide were examined at the border areas using a defined rectangular field area with a magnification of x20. The apoptosis rate of cardiomyocytes was presented as the percentage of total cells counted.

**Assessment of serum cardiac enzymes.** At the end of myocardial reperfusion and prior to sacrifice, 3-5 ml blood samples were collected from the abdominal aorta. Subsequently, the blood samples were centrifuged at 2,000 x g for 5 min at room temperature, and the serum obtained was assayed for the activity of cardiac enzymes [creatine kinase (CK), CK-MB isoenzyme and lactate dehydrogenase (LDH)]. The activity levels of CK, CK-MB and LDH were detected using the creatine kinase assay kit (cat. no. A032), creatine kinase MB isoenzyme assay kit (cat. no. E006) and lactate dehydrogenase assay kit (cat. no. A020-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively, in accordance with the manufacturer's protocols using an automatic biochemical analyzer.

**Western blotting.** Heart tissues were lysed in radioimmuno-precipitation assay lysis buffer (EMD Millipore, Billerica, MA, USA) containing complete™ Protease Inhibitor Cocktail (CWBio, Beijing, China) and homogenized with glass homogenizer until full lysis was achieved. Cell lysates were obtained by centrifuging homogenates at 6,000 x g for 10 min at 4°C. Protein concentration was determined using a standard bicinchoninic acid assay. Total protein (10 µg/lane) was separated using SDS-PAGE (10% gels) and then transferred onto nitrocellulose membranes. The membranes were incubated with anti-cleaved Caspase-8 (C-Caspase-8; cat. no. 66093-1-Ig), anti-cleaved Caspase-3 (C-Caspase-3; cat. no. 19677-1-AP), anti-B-cell lymphoma 2 (Bcl-2; cat. no. 12789-1-AP), anti-Bcl2-associated X protein (Bax; cat. no. 50599-2-Ig), anti-cleaved poly(ADP-ribose) polymerase (C-PARP; cat. no. 13371-1-AP), all 1:2,000 and anti-GAPDH (cat. no. 60004-1-Ig; 1:10,000; all Wuhan SANYING Biotechnology, Wuhan, China) primary antibodies overnight at 4°C. Following this, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (cat. no. ab6721) and rabbit anti-mouse (cat. no. ab6728) secondary antibodies (1:5,000; Abcam, Cambridge, MA, USA). Blots were analyzed using an electrochemiluminescence detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Biochemical assays.** To assess the extent of oxidative stress, the activity of GPx and SOD, and levels of malondialdehyde (MDA) and protein carbonyl (PC) in heart tissue were measured. Heart tissue samples were homogenized in PBS, and the supernatant was obtained by centrifuging at 6,000 x g for 10 min at 4°C and preserved at -80°C until analysis. GPx and SOD activity levels were detected using the methods described by Paglia and Valentine (19) and Snow-Lisy et al (20), respectively, and enzyme activity was recorded as U/g of protein in heart tissue. MDA levels were measured using a thiobarbituric acid reaction, which was described by Ohkawa et al (21). The assay used spectrophotometric measurements of the color produced during the reaction of thiobarbituric acid with MDA at 535 nm, which were then expressed as nmol/mg of protein. PC levels were measured spectrophotometrically according to the reaction of the carbonyl group with 2, 4-dinitrophenylhydrazine, which is used as a reagent for proteins subjected to metal-catalyzed oxidation as described in detail by Levine et al (22). The result was recorded as nmol carbonyl/mg of protein.

**Statistical analysis.** Data were expressed as mean ± standard error of the mean and analyzed using SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed using one-way analysis of variance followed by a Student-Newman-Keuls post-hoc test for multi-group comparisons. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Ebselen protects against myocardial I/R injury and cardiac dysfunction.** To detect whether ebselen protects against myocardial I/R injury, rats were administered ebselen intragastrically prior to the I/R-inducing operation. Rat hearts were subjected to ischemia for 30 min, which was followed by reperfusion for 2 h. Subsequently, hearts were harvested for infarct size evaluation. As depicted in Fig. 1A, the infarct size was significantly increased in the I/R group compared with in the sham group (P<0.01), which was indicated by the ratio of IA/AAR. Meanwhile, in the ebselen + I/R group, the infarct size was reduced compared with in the I/R group (P<0.05). Additionally, the effect of ebselen on cardiac function was evaluated following myocardial I/R injury via echocardiography. As demonstrated in Fig. 1B and C, the EF% and %FS were significantly decreased in the I/R group compared with in the sham group at 7 days post I/R injury induction (P<0.05). EF% and %FS were increased in the ebselen + I/R group compared with in the I/R group (P<0.05), suggesting ebselen protected against the cardiac dysfunction induced by myocardial I/R injury.

Histopathological and ultrastructural changes of the myocardium were observed using light microscopy (H&E staining) and electron microscopy. As shown in Fig. 2A, myocardial fibers exhibited an orderly arrangement and no evidence of inflammatory cell infiltration was evident in the sham and ebselen control groups. Meanwhile, focal necrosis of the myocardium, myocardial cell swelling, cell boundary and transverse stripe blur, and neutrophil infiltration in the interstitial area and between cardiomyocytes were observed in the I/R group. In the ebselen + I/R group, these changes were markedly attenuated compared with in the I/R group. However, the myocardial cells still exhibited some swelling and a small amount of inflammatory cell infiltration was observed in the ebselen + I/R group. Ultrastructural changes to the organelles in the cytoplasm were observed using electron microscopy. As demonstrated in Fig. 2B, in the sham and ebselen control groups, closely and uniformly arranged myocardial fibers, clear muscle segments, abundant mitochondria and some vacuoles in the cytoplasm were observed. In the I/R group, notable mitochondrial swelling induced by myocardial I/R injury was observed along with abundant fatty and vacuolation degeneration in cardiomyocytes compared with in the sham group.
The arrangement of myocardial fibers became disordered and damage of myocardial fibrous membrane was also observed. In the ebselen + I/R group, these changes were reduced and the cardiac cellular structure remained clearly visible.

**Ebselen attenuates I/R-induced myocardial apoptosis and cardiac injury.** Narula et al (23) reported that cardiomyocyte apoptosis contributes to myocardial I/R injury. In the current study, it was determined whether ebselen serves an anti-apoptotic role during myocardial I/R injury. TUNEL staining (Fig. 3A) indicated that I/R injury significantly induced myocardial apoptosis by 29% compared with sham treatment (P<0.01). Furthermore, the rate of myocardial apoptosis in the ebselen + I/R group was reduced compared with the I/R group (P<0.05). The activity of the cardiac enzymes CK, CK-MB and LDH in serum was also evaluated. As indicated in Fig. 3B-D, the activity levels of serum CK, CK-MB and LDH were markedly increased in the I/R group compared with in the sham group (P<0.05). In turn, ebselen significantly attenuated the activity levels of CK, CK-MB and LDH compared with I/R alone (all P<0.05).

**Ebselen influences the expression of apoptotic proteins.** To clarify the mechanisms of ebselen in inhibiting cardiomyocyte apoptosis, the expression of the apoptosis-related proteins C-Caspase-8, C-Caspase-3 and C-PARP was assessed. Western blotting (Fig. 4) demonstrated that I/R injury significantly increased the expression of myocardial pro-apoptotic proteins C-Caspase-8, Bax (both P<0.01), C-Caspase-3 and C-PARP, and decreased the expression of the anti-apoptotic protein Bcl-2 (all P<0.05) when compared with sham treatment. By contrast, treatment with ebselen suppressed the expression of pro-apoptotic proteins in heart tissues and significantly promoted the expression of Bcl-2 compared with I/R alone (all P<0.05).

**Effects of ebselen on the levels of cardiac antioxidant enzymes, MDA and PC.** The effect of ebselen on cardiac antioxidation during I/R injury was evaluated. As indicated in Fig. 5A and B, SOD and GPx activity levels were decreased in the I/R group compared with in the sham group (P<0.01). In the ebselen + I/R group, the antioxidant enzyme activities were significantly increased compared with in the I/R group (P<0.05). MDA is the final product of lipid peroxidation. MDA levels, as a marker of cardiac oxidative stress, were assayed in heart tissues in the present study. As indicated in Fig. 5C, I/R injury significantly increased the MDA levels in the heart tissues compared with in the sham group (P<0.05). This I/R-induced increase in MDA levels was significantly prevented by ebselen treatment (P<0.05). PC levels in heart tissues were also determined (Fig. 5D). A marked increase in the tissue levels of PC was observed in the I/R group (P<0.05 vs. sham), suggesting increased protein oxidation. Meanwhile, PC levels were significantly decreased in the ebselen + I/R group compared with in the I/R group (P<0.05).

**Discussion**

Coronary reperfusion therapy is an established approach for the management of acute myocardial infarction; however, restoration of blood flow to previously ischemic myocardium results in I/R injury (24,25). Cumulative studies have uncovered that several primary factors, including oxidative stress, intracellular calcium overload, fluctuating physiological pH, the mitochondrial permeability transition pore and inflammation mediate the detrimental effects of myocardial I/R injury. These factors collectively induce apoptosis and the death of cardiomyocytes during the I/R process (26-29). Therefore, reducing cardiomyocyte apoptosis may protect the heart against I/R-induced injury.

A number of studies have uncovered that ebselen has beneficial effects in I/R-induced tissue injury, specifically in intestinal (30), lung (31), kidney (32) and sciatic nerve (33) tissues. In the current study, the effects of ebselen on heart injury induced by I/R in vivo were examined. The major findings included the following: i) Ebselen significantly attenuated I/R-induced myocardial infarction and cardiac dysfunction; ii) ebselen attenuated the effects of I/R in reducing SOD and GPx activities and increasing MDA and PC levels in rat hearts, consistent with results reported by Baljinnyam et al (34) in a rabbit model of myocardial I/R injury; iii) ebselen prevented I/R-induced rat heart injury, by attenuating histological and ultrastructural changes, reducing serum CK, CK-MB and LDH activity levels, and decreasing cell apoptosis, which to our knowledge is the first time this has been systematically determined; and iv) ebselen pretreatment markedly decreased the expression of C-Caspase-8, C-Caspase-3, Bax and C-PARP, and increased the expression of Bcl-2.
In myocardial ischemia, hypoxia and reoxygenation, as principal causes of reperfusion injury, induce an increase in free radical production in cardiac tissues (35,36). ROS and RNS produced through reoxygenation lead to direct oxidative damage. Ebselen attenuates I/R-induced myocardial apoptosis and cardiac injury. (A) Myocardial apoptosis was examined using the TUNEL assay in rat heart sections. Representative images of TUNEL staining (green fluorescence) were captured under a fluorescence microscope. The bar graph indicates the rate of myocardial apoptosis. (B-D) Effects of ebselen on serum cardiac enzymes CK, CK-MB and LDH were demonstrated. Each bar represents the mean of three independent experiments. Data are expressed as the mean ± standard error of the mean. **P<0.01 and *P<0.05 vs. sham group; #P<0.05 vs. I/R group.

I/R, ischemia/reperfusion; E, ebselen; TUNEL, terminal dUTP nick end-labeling; CK, creatine kinase; CK-MB, creatine kinase MB isoenzyme; LDH, lactate dehydrogenase.
damage to cellular components; in particular, damage caused by peroxidation of cellular membrane lipids and oxidation of cellular proteins, which can be measured by assessing MDA and PC levels in the heart tissue, respectively. The present results indicated that I/R injury caused a significant increase in MDA and PC levels in the heart tissues. However, this change was mostly restored in the ebselen-pretreated I/R group. Additionally, it was examined whether ebselen could enhance the antioxidant capacity of rat hearts in the current study. It was identified that myocardial I/R injury decreased SOD and GPx activities, and this effect was attenuated by treatment with ebselen. The effects of ebselen on SOD and GPx activities have been excessively studied in myocardial I/R injury in several animal species including rabbit, in situ swine and neonatal porcine models (34,37-39), and the current results in a rat model are consistent with those of the previous studies.
However, there appears to be few reports that have determined an increase in MDA and PC levels in myocardial I/R injury, and that ebselen may decrease these levels. Additionally, further investigation is needed to elucidate the role of SOD and GPx in the ebselen-mediated cardioprotection during I/R injury and the anti-oxidant effects of ebselen in myocardial I/R injury, potentially occurring via nitric oxide signaling, in future studies.

A key finding of the present study was that ebselen exhibited various I/R-protective effects via antioxidant and anti-apoptosis functions. Previous studies have indicated that apoptosis induced by oxidative stress serves an important role in the pathogenesis of heart dysfunctions induced by myocardial I/R injury (40-42). Several critical factors, including C-Caspase-8, C-Caspase-3, Bax, Bel-2 and C-PARP, regulate the process of apoptosis (43-47). In myocardial I/R injury, inhibition of C-Caspase-8, C-Caspase-3 and C-PARP may reduce cardiomyocyte apoptosis (43,44). Furthermore, previous results indicated that overexpression of Bcl-2 and downregulation of Bax inhibited myocyte apoptosis following reperfusion, and protected the heart against I/R injury (45-47).

In the current study, the protein expression of genes associated with cell apoptosis was detected using western blotting. According to the results, C-Caspase-8, C-Caspase-3, Bax and C-PARP were overexpressed in the I/R group compared with the sham group; in turn, the protein expression of these pro-apoptotic factors was decreased in the ebselen-pretreated I/R group. By contrast, the expression of Bel-2 in rat hearts was decreased in the I/R group compared with the sham group, and subsequently increased by ebselen pretreatment compared with the I/R group. To the best of our knowledge, the present study is the first to indicate that ebselen protects against myocardial I/R injury by upregulating Bel-2 and downregulating C-Caspase-8, C-Caspase-3, Bax and C-PARP, thus inhibiting I/R-induced apoptosis.

In conclusion, the present study indicated that ebselen could protect rat hearts against I/R injury. Ebselen attenuated I/R-induced cardiomyocyte apoptosis seemingly by increasing the expression of Bel-2, decreasing the expression of C-Caspase-8, C-Caspase-3, Bax and C-PARP, and enhancing the expression and function of antioxidant enzymes. Taken together, the present results support the use of ebselen as an effective therapeutic agent for the treatment of myocardial I/R injury.

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Availability of data and materials

Please contact the corresponding author for data requests.

Authors' contributions

BC and JPZ performed the animal and molecular studies, collected experimental data and drafted the manuscript. GLL, QXR and GL participated in the design of the study and performed the histological examination of the hearts and the statistical analysis. FXW and HJ conceived the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Laboratory Animal Ethics Committee of Wuhan University (Wuhan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


Please contact the corresponding author for data requests.


