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Do N-Acetylcystein, β-Glucan, and Coenzyme Q10 Mollify Myocardial Ischemia-Reperfusion Injury?

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ABSTRACT

Background. N-acetylcysteine, β-glucan, and coenzyme Q_{10} have been shown to have antioxidant and anti-inflammatory effects on reperfusion injury. The aim of our study was to determine and evaluate the effects of these agents on myocardial ischemia-reperfusion injury.

Methods. Forty-four New Zealand white rabbits, all female, weighing 2.4 to 4.1 kg (mean, 3.6 kg) were used in the study. Four study groups of 11 animals were arranged by randomization. The groups were the control group (group C), a group premedicated with coenzyme Q_{10} (group Q), a group premedicated with β-glucan (group βΓ), and a group premedicated with N-acetylcysteine (group N). After exploration of the heart, a basal myocardial biopsy was taken from the anteroapical left ventricle, and the first blood sampling was done before ischemia. For the ischemia-reperfusion experiments, the major left anterior descending artery was occluded after baseline measurements. After a 45-minute transient ischemic period, the heart was perfused for 120 minutes. After perfusion, the second myocardial biopsy was taken from the anteroapical left ventricle, and the second blood sampling was done. Blood and tissue analysis were performed and evaluated statistically.

Results. Baseline and reperfusion levels of glutathione peroxidase, superoxide dismutase, malonyldialdehyde, and nitric oxide changed significantly. While malonyldialdehyde levels increased in group C, they decreased in the other study groups ($P = .001$). The increases in glutathione peroxidase and superoxide dismutase levels were significant in all groups except group C ($P = .0001$ and $P < .05$, respectively). Levels of nitric oxide were found to be decreased in group C, whereas they increased in the other groups ($P = .001$).

Conclusion. Antioxidant medication may help in lowering the risk of myocardial ischemia-reperfusion injury. All the medications in our study are shown to have effective roles in preventing ischemia-reperfusion injury to some extent through their antioxidant properties.

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INTRODUCTION

The restoration of myocardial blood flow after an ischemic period may increase the severity of the ischemic tissue injury. There are several studies that show that reintroduction of molecular oxygen into ischemic tissue upon reperfusion leads to excessive formation of reactive oxygen species (ROS), which may overwhelm the tissue antioxidant defense capacity and damage myocardial cells [Park 1999].

One of the cellular defense mechanisms against ROS is the enzymatic part represented by free-radical scavenger enzymes, namely superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). Another mechanism is the nonenzymatic part including a large number of natural or synthetic antioxidant compounds that have the ability to inhibit the oxidative damage by scavenging the highly destructive free-radical species such as glutathione, vitamin C, and vitamin E [Naranjan 2000].

Activated neutrophils, stressed cardiomyocytes, activated vascular endothelium, and, to a minor extent, perivascular tissue are the sources of ROS. Vascular endothelial cells have several enzyme systems that generate ROS, such as the endothelial nicotineamide adenine dinucleotide phosphate (NADPH) oxidase system, xanthine oxidase, and endothelial nitric oxide (NO) under some conditions. Neutrophil-endothelial interactions during the first minutes of reperfusion lead to activation of the local inflammatory cascade, which contributes to endothelial cell dysfunction, necrosis, and perhaps apoptosis. However, as a result of neutrophil-dependent and neutrophil-independent actions, endothelial cells and cardiomyocytes generate oxidants that contribute to necrosis [Jennings 1960; Marchioli 1999; Park 1999; Naranjan 2000].

The healthy vascular endothelium tonically releases NO by endothelial NO synthase activity. However, the release of NO by coronary vascular endothelium is impaired after ischemia-reperfusion. NO may be involved both in inhibiting inflammatory responses and molecular responses to ischemia-reperfusion [Jennings 1960; Schaper 1997; Marchioli 1999].

Of the several medications used to limit ischemia-reperfusion injury, the antioxidants are the most forthcoming. N-acetylcysteine (NAC), a thiol-containing compound, has been shown to have cytoprotective and microcirculatory effects and restores the cellular antioxidant potential by replenishing depleted glutathione stores. It is a scavenger of oxygen radicals both directly and as a precursor of glutathione and inhibits the neutrophil aggregation [Orhan 2006].

Coenzyme Q_{10} (CoQ_{10}) is an essential component for electron transportation in oxidative phosphorylation of mitochondria. Also called ubiquinone, its principal function is to act as an electron carrier between the NADH and succinate dehydrogenases and the cytochrome system [Menasche 1992]. It appears to have clinically relevant antioxidant properties manifested by tissue protection in settings of ischemia and reperfusion. CoQ_{10} appears to be involved in the coordinated regulation between oxidative stress and antioxidant capacity of heart tissue. When the heart is subjected to oxidative stress in various pathogenic conditions, the amount of CoQ_{10} is decreased, which triggers a signal for increased CoQ_{10} synthesis [Sunamori 1991; Menasche 1992; Chello 1994].

β-glucan, derived from broken cell walls of yeast, mushrooms, barley, and oats, is capable of reducing unhealthy amounts of serum cholesterol and boosting the immune system. β-glucan is a powerful immune stimulator, activating the macrophage in the immune system. Studies have found that this product not only has a positive action on the macrophage, but also on B-lymphocytes, natural killer cells, and suppressor T-cells. In addition to these important functions, β-glucan is an effective antioxidant and free-radical scavenger [Rosenfeldt 2005].

Each of these agents were subjected to several studies and were shown to have antioxidant and anti-inflammatory effects. The aim of our study was to determine and compare the effects of these agents in an in situ model of acute coronary occlusion.

METHODS

The experiment was performed in compliance with the Principles of Laboratory Animal Care formulated by the National Institutes of Health (National Institutes of Health publication no. 96 to 23, revised 1996). The experiment and animal care protocol was approved by the Ethics Committee for Animal Care, established in our institute.

Forty-four female New Zealand white rabbits, weighing 2.4 to 4.1 kg (mean, 3.6 kg) were used in the study. Four study groups of 11 animals were arranged by randomization. Groups were named according to the medication given for prophylaxis.

The groups were the control group (group C), a group premedicated orally with 15 mg/kg CoQ_{10} (Sigma Chemical, St. Louis, MO, USA) 2 times a day for 10 days (group Q), a group premedicated with 2 mg/kg β-glucan (Mustafa Nevzat Drug, Istanbul, Turkey) via an intraperitoneal route for 10 days (group βG), and a group premedicated with intravenous 50 mg/kg NAC (Husnu Arsan Drug, Istanbul, Turkey) perfusion just before the operation (group N).

After premedication with intramuscular 0.05 mg/kg midazolam, 50 mg/kg intramuscular ketamine administration was used. Pentobarbital anesthesia was given during the study as required to maintain a deep level of anesthesia. The rabbits were intubated and mechanically ventilated. Surface 4-lead extremity electrocardiogram was recorded continuously. The right carotid artery was catheterized for invasive blood pressure monitoring, and auricular vein cannulation was performed for venous sampling and NAC infusion in group N. Following cannulation of the external jugular vein, animals received a continuous infusion of normal saline of 15 mL/kg per hour to compensate for fluid losses.

Sternotomy was carefully performed, avoiding opening the pleura. After a careful exploration of the heart, the first blood sampling and basal myocardial biopsy were taken from the anteroapical left ventricle with a biopsy needle (gauge 4) after a 10-minute stabilization period. For the ischemia-reperfusion experiments, after baseline measurements, the major left anterior descending artery was found and encircled with a 4-0 silk suture. The ends of the suture were threaded thorough a piece of tubing, forming a snare that was tightened to occlude the artery. The effectiveness of this maneuver was verified by the appearance of epicardial cyanosis, electrocardiographic changes, and myocardial enzyme changes. Ventricular fibrillation during coronary occlusion was treated by an electrical defibrillation (5 J defibrillator). After 45 minutes of occlusion, the snare occluder was released and the artery was reperfused. Reperfusion was verified by the disappearance of epicardial cyanosis and normalization of the electrocardiographic changes. During the ischemic period, some deteriorations in hemodynamic status occurred, but severe instability did not occur in any of the rabbits. After the transient ischemic period, the heart was perfused for 120 minutes. At the end of the reperfusion period, the second myocardial biopsy was taken from the anteroapical left ventricle. The blood sampling (2 mL) was done from the right atrium after sternotomy before ischemia and after the reperfusion period. Arterial blood gases were monitored during the ischemia-reperfusion experiment.

After these processes, the animals were sacrificed by intravenous administration of a high concentration of pentobarbital.

Blood and Tissue Sample Analysis

The blood samples were collected in glass tubes containing additives and centrifugated at 4°C with 3000 rpm for 5 minutes. Plasma was removed into tubes and stored at –70°C until measurement. Erythrocytes were washed 3 times with 2 volumes of isotonic saline. Then erythrocytes were lysed with cold distilled water (1:4), stored in a refrigerator at 4°C for 15 minutes, and centrifugated at 4°C at 2000 rpm for 15 minutes. Supernatant fluids were removed and stored at –70°C until measurement. Plasma samples were used for measurement of nitrite-nitrate and malonyldialdehyde (MDA) levels. Erythrocyte lysates were used for measurement of GPx and copper-zinc SOD activities. The tissue

samples were stored in tubes not containing additives at –70°C. These samples were homogenized in cold potassium chloride solution (1.15%) in a glass homogenizer and centrifuged at 4°C at 5000*g*. The supernatant fluid was removed and used for GPx and SOD activity and nitrite-nitrate and MDA level measurements.

GPx

GPx activities in tissue supernatant and erythrocyte lysates were measured using the method described by Pleban et al [1982]. The reaction mixture was 50 mmol/L tris buffer, pH 7.6 containing 1 mmol/L of Na_2EDTA, 2 mmol/L of reduced glutathione, 0.2 mmol/L of NADPH, 4 mmol/L of sodium azide, and 1000 U of glutathione reductase, 50 µL of erythrocytes and 950 µL of reaction mixture or 20 µL of tissue supernatant fluid and 980 µL of reaction mixture were mixed and incubated for 5 minutes at 37°C. Then the reaction was initiated with 8 mmol/L H_2O_2, and the decrease in NADPH absorbance was fallowed at 340 nm for 3 minutes. Enzyme activities were expressed as U/g in tissue measurements and U/mL in serum measurements.

Copper-Zinc SOD

SOD activity in tissue supernatant and erythrocyte lysates were measured using the method described by Fitzgerald et al. Briefly, each supernatant and erythrocyte lysate was diluted 1:400 with 10 mM phosphate buffer, pH 7.00. Twenty-five µL of diluted supernatant and erythrocyte lysate was mixed with 850 µL of substrate solution containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride in a buffer solution containing 50 mmol/L CAPS and 0.94 mmol/L EDTA pH 10.2. Then, 125 µL of xanthine oxidase (80 U/L) was added to the mixture and absorbance increase was followed at 505 nm for 3 minutes against air. Twenty-five µL of phosphate buffer or 25 µL of various standard concentrations in place of the sample were used as blank or standard determinations. Copper-zinc SOD activity was expressed as U/g in tissue measurements and U/mL in erythrocyte measurements.

Nitrite/Nitrate

Plasma and tissue homogenate NO_2^-/NO_3^- concentrations were determined by using the Griess reaction. The reaction mixture consisted of reduced NADPH, flavin adenine dinucleotide, and nitrate reductase. After incubation of plasma and tissue supernatant samples with reaction mixture, Griess reagent (a 1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% N-[1-naphtyl]-ethylenediamine) was added to the samples. After incubating for 10 minutes, the absorbance was measured spectrometrically at 540 nm. The nitrite/nitrate concentrations in the samples were calculated from the standard curve [Aydin 2001].

Lipid Peroxidation Assay for MDA

MDA levels were determined as described by Ohkawa et al [1979]; 100 µL of plasma or tissue was added to 50 µL of 8.1% sodium dodecyl sulfate, vortexed, and incubated for 10 minutes at room temperature. Three hundred seventy-five µL of 20% acetic acid (pH 3.5) and 375 µL of thiobarbituric acid (0.6%) were added and placed in a boiling water bath for 60 minutes. The samples were allowed to cool at room temperature before 1.25 mL of butanol pyridine (15:1) was added, vortexed, and centrifuged at 1000 rpm for 5 minutes. Seven hundred fifty µL of the organic pink layer was measured at 532 nm. As a standard, 1,1,3,3-tetraethoxypropane was used.

Statistical Analysis

Statistical analyses were done by a SPSS (Chicago, IL, USA) statistical program. Measured data are expressed as mean values ± standard deviation. Wilcoxon test was used for the comparison of measurements before and after the ischemic period. A P value < .05 was considered significant.

RESULTS

The blood pressure and heart rate monitoring showed deterioration during the ischemic period and recovered with the reperfusion. The hemodynamic data are provided in the Table. Despite deterioration in group C, no significant changes were seen in the medicated groups. Although there was a statistically significant difference between the control and medicated groups for hemodynamic parameters ($P < .05$), no significant changes were detected among the medicated groups ($P > .05$). The baseline (before ischemia) and postreperfusion levels of serum and tissue GPx, SOD, MDA, and NO are detailed in Figures 1 and 2. The increase in GPx and SOD levels in reperfused tissue were significant in the medicated groups when compared to group C. The increase in blood GPx levels was most significant in the βG and N groups. The change in blood SOD levels showed a more

Changes in Hemodynamic Parameters (Systolic Blood Pressure [BP] and Heart Rate [HR]) during the Ischemia-Reperfusion Period*

	Baseline		Ischemia		Reperfusion	
	BP	HR	BP	HR	BP	HR
Group C	113 ± 12	88 ± 4	84 ± 4	123 ± 3	94 ± 4	99 ± 4
Group N	104 ± 9	86 ± 5	103 ± 3	76 ± 2	112 ± 5	81 ± 3
Group Q	108 ± 7	83 ± 5	102 ± 4	72 ± 4	105 ± 4	78 ± 3
Group βG	113 ± 8	84 ± 6	105 ± 2	78 ± 5	109 ± 3	82 ± 4

*BP was measured in mmHg; HR, beats per minute. Group C indicates the control group; Group N, N-acetylcystein group; Group Q, coenzyme Q10 group; Group βG, β-glucan group.

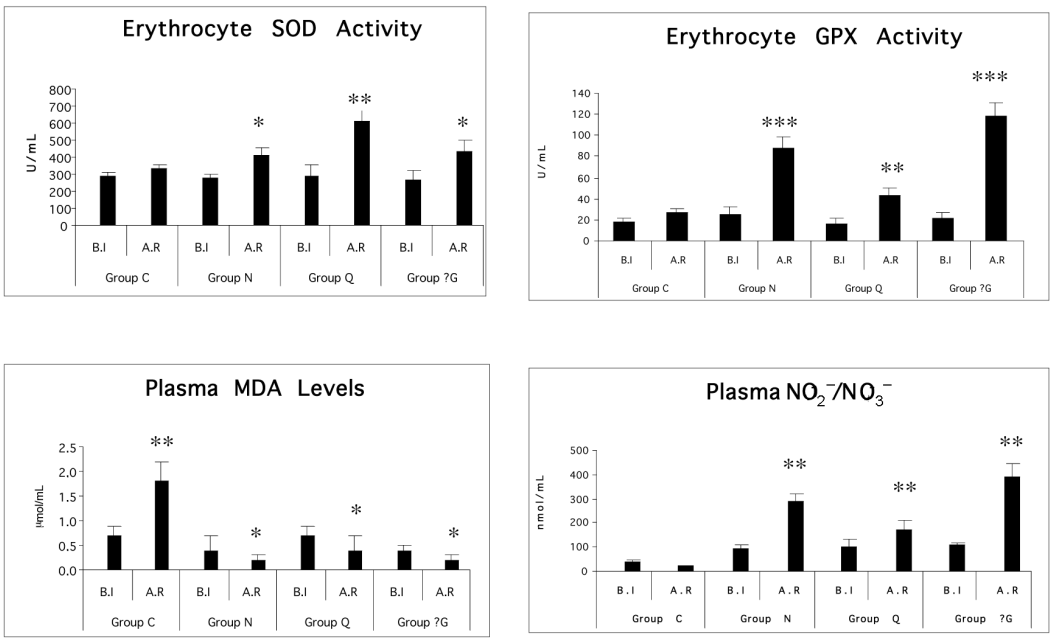

Figure 1. Blood oxidative stress status and nitric oxide levels of rabbits given antioxidant supplements after reperfusion injury. Group C indicates the control group; Group N, N-acetylcystein group; Group Q, coenzyme Q10 group; Group bG, b-glucan group; BI, before ischemia; AR, after reperfusion. *P < .05. **P < .001. ***P = .0001.

significant increase in group N. While the MDA levels increased in group C, they were found to be decreased in the groups treated with antioxidants. Increase in blood NO levels was most prominent in group βG.

In the control group, GPx, SOD, and MDA increased and NO_2^-/NO_3^- decreased following reperfusion. Increase in the antioxidant enzymes demonstrates the severity of the toxic radical injury. After reperfusion, increasing enzyme levels

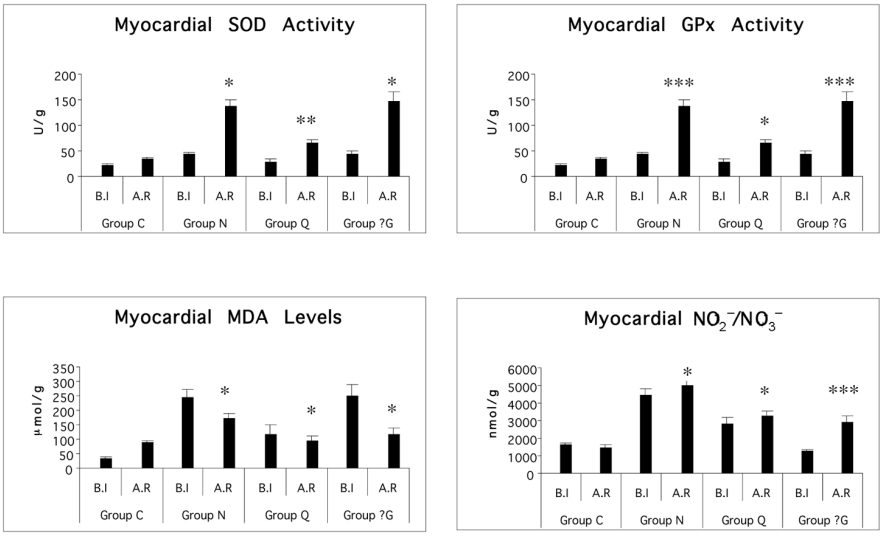

Figure 2. Myocardial oxidative stress status and nitric oxide levels of rabbits given antioxidant supplements after reperfusion injury. Group C indicates the control group; Group N, N-acetylcystein group; Group Q, coenzyme Q10 group; Group βG, β-glucan group; BI, before ischemia; AR, after reperfusion. *P < .05. **P < .001. ***P = .0001.

tried to prevent tissue injury. Despite this increase, they were unable to decrease the MDA, the marker of membrane injury. When compared to the control group, antioxidant enzyme levels increased significantly in the medicated groups. The NAC behaved like a cystein source for the cells. All of the medications were enough for the enzymes and predominantly decreased the MDA and increased the NO_2^-/NO_3^-. From this point of view, they may be postulated as protective against ischemia-reperfusion injury. Also, NO_2^-/NO_3^- levels increased significantly in the medicated groups. We may also say that these 3 antioxidant agents may be beneficial for the regulation of endothelial functions.

DISCUSSION

Ischemia-reperfusion injury is a complex inflammatory process, based on the reintroduction of fully oxygenated blood into the targeted areas of myocardium previously modified by ischemia, usually seen as decreased cardiac functions in the postoperative period. Endothelial cells, neutrophils, the complement system, cytokines, and ROS such as superoxide anion, hydrogen peroxide, and hypochlorus acid play a major role in this process [Park 1999].

Free-oxygen radicals can cause several damages by DNA strand breaking, lipid peroxidation, neutrophil recruitment, inactivation of NO, and degradation of proteins such as antioxidant and antiprotease enzymes [Jennings 1960; Schaper 1997; Marchioli 1999; Park 1999; Naranjan 2000; Orhan 2006].

Many substances such as antioxidants and/or free-radical scavengers have been shown to minimize reperfusion injury and indirectly support the key role played by oxygen radicals in reperfusion injury [Park 1999; Orhan 2006].

MDA levels indicate an amount of cellular damage secondary to lipid peroxidation and have been widely adopted as a measure of free-radical formation. In our study, the MDA levels in the control group were significantly increased after ischemia-reperfusion. In our medicated groups, the levels were found to be decreased, which may show the effects of antioxidant medication on limiting ischemia-reperfusion injury [Tracey 1995; Park 1999; Naranjan 2000].

Today, NAC is perceived as a drug that is applicable for acute coronary syndromes. NAC directly eliminates hydroxyl radicals and increases the NO system-dependent coronary flow. This is an advantage when using NAC as a part of cardioplegic solutions. Evidence is also available that NAC acts during cardiopulmonary bypass as an oxygen-radical scavenger and also stabilizes neutrophils in relation to their oxidative response to the bypass [Ohkawa 1979; Ceconi 1988; Tang 1991; Menasche 1992; Andersen 1995; Sochman 2002].

In our study, NAC seemed to limit reperfusion injury. In groups medicated with NAC, all antioxidant defense markers were significantly increased. The most significant increase was in GPx. Our results were similar to findings in the above-mentioned studies that indicate NAC enhances GPx and SOD activity and helps the modulation of damaged endothelium and NO release.

Ischemic heart disease, anginal syndromes, and most recently the ischemia-reperfusion injury of coronary revascularization has provided clear evidence of clinically relevant antioxidant cell-protective effects of CoQ_{10}. Several reports exist in the literature that indicate cardioprotective effects of CoQ_{10} against ischemia-reperfusion injury [Sumanori 1991; Menasche 1992; Chello 1994].

In 1994, Chello et al randomized 40 patients to receive either placebo or 150 mg/day of oral CoQ_{10} 1 week prior to coronary artery bypass graft surgery. A significant decrease in postoperative markers of oxidative damage was observed in the treatment group with lower concentrations of coronary sinus thiobarbituric acid reactive substances, conjugated dienes, and cardiac isoenzymes of creatine kinase.

CoQ_{10} appears to be involved in the coordinated regulation between oxidative stress and antioxidant capacity of heart tissue. When the heart is subjected to oxidative stress in various pathogenic conditions, the amount of CoQ_{10} is decreased, which triggers a signal for increased CoQ_{10} synthesis [Mortensen 1996; Cotgreave 1997].

In our study, we found out that CoQ_{10} limits reperfusion injury mostly via enhancing SOD and GPx activity. These results were compatible with the results from several studies designed to determine the effects of CoQ_{10} on reperfusion injury.

The correlation of the dynamics of ROS formation with the time course of GPx activity as well as the antioxidant vitamin levels was followed to better understand the consequences of reperfusion after acute myocardial infarction. The reintroduction of molecular oxygen into ischemic tissue upon reperfusion has been proven to lead to excessive formation of ROS, which may overwhelm the tissue antioxidant defense capacity and damage myocardial cells. Ample evidence exists suggesting that free radicals are already produced to a limited extent during myocardial ischemia with a marked increase during the phase of reperfusion. During myocardial ischemia, suppressed levels of the natural defense system were also detected [Marchioli 1999; Park 1999; Naranjan 2000].

GPx was found to play a crucial role in myocardial protection from ischemia-reperfusion injury. Decreased GPx activity in human cardiomyocytes was found to be consistent with increased susceptibility to oxidant injury, while overexpression of the gene for GPx made the mouse heart more resistant to myocardial infarction-reperfusion injury [Marchioli 1999; Park 1999; Naranjan 2000].

Endothelial cells in reperfused myocardium assume an activated state in which they express adhesion proteins, release cytokines, and reduce production of NO. This promotes adherence, activation, and accumulation of neutrophils and monocytes in the ischemic-reperfused tissue. The release of ROS and proteolytic enzymes from these activated leukocytes can contribute to the damage of myocytes and vascular cells [Jennings 1960; Marchioli 1999; Park 1999; Naranjan 2000].

β-glucan, derived from broken cell walls of yeast, mushrooms, barley, and oats, is capable of reducing unhealthy amounts of serum cholesterol and boosting the immune system. β-glucan is a powerful immune stimulator, activating the macrophages and complement system [Rosenfeldt 2005].

β-glucan is an effective antioxidant and free-radical scavenger [Chew 2004; Rosenfeldt 2005].

Our results were similar to the findings about β-glucan demonstrated by other studies. β-glucan was found to increase both GPx and SOD levels and NO release. As a result, a significant decrease in MDA was observed.

When we evaluated the medications, all were found to be effective in limiting reperfusion injury. NAC and CoQ_{10} were mostly effective in limiting the injury by enhancing the GPx and SOD activity and to a lesser extent by modulating endothelial damage and NO release. On the other hand β-glucan was found to have both antioxidant and endothelial protective effects.

CONCLUSION

Antioxidant medication may help in lowering the risk of myocardial ischemia-reperfusion injury. According to our results, all medications in this study, to some extent, may be useful for preventing the negative effects of ischemia-reperfusion injury through their antioxidant properties. From a clinical point of view, ischemia-reperfusion injury is still a challenging complication. Any drug that prevents this injury will be of great value. It can be postulated that the medications studied in our research may be used to prevent ischemia-reperfusion injury, but further investigation of the potential effects of this cardioprotective strategy is needed.

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