Morinda Citrifolia (Noni) and Low Dose Aspirin Prevent Apoptotic Cell Death and Oxidative Stress on Isoproterenol Induced Myocardial Infarction in Rats

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ABSTRACT

Objective: Isoproterenol (iso)-induced myocardial infarction is a widely used experimental model to study the beneficial effects of many drugs. Morinda citrifolia (noni) has traditionally been used for treating many diseases. Low-dose aspirin may have been protected heart tissue. This study aimed to investigate the possible protective effects of noni (20%) and low-dose aspirin (30 mg/kg) against iso-induced acute myocardial infarction in male Wistar rats.

Materials and Methods: Rats were divided into control, iso, iso+noni, iso+aspirin, and iso+aspirin+noni groups (n=8). Myocardial infarction was induced by subcutaneous injection of iso (100 mg/kg) for two consecutive days. Iso-induced rats showed increased serum Creatine kinase- muscle brain CK-MB activity. The effects of noni (20%) and low-dose aspirin (30 mg/kg) were evaluated in response to iso-induced changes, Malondialdehyde (MDA), Superoxide Dismutase (SOD), Catalase (CAT), DNA fragmentation, caspase-3, and cathepsins B and L.

Results: Oral treatment with 20% noni juice and low-dose aspirin (30 mg/kg) for 7 days significantly decreased apoptotic markers such as DNA fragmentation, caspase-3, and cathepsins B and L; increased antioxidant enzymes such as SOD and catalase activities; and decreased MDA levels.

Conclusion: We propose that the protective effect, antiapoptotic and antioxidant mechanisms of noni and low dose aspirin may be on iso-induced myocardial infarction.

Keywords: Aspirin, cathepsin B, caspase-3, catalase, Morinda citrifolia (noni), SOD

INTRODUCTION

Myocardial infarction is one of the main causes of death from cardiovascular diseases. Myocardial ischemia occurs when myocardial oxygen supply is less than myocardial oxygen demand; thus, it causes cell injury known as myocardial infarction (1).

Excessive endogenous release or exogenous administration of catecholamine such as isoproterenol (iso), a β-adrenergic agonist, causes severe stress in the myocardium, resulting in infarct-like necrosis of the cardiac muscles. This leads to complex biochemical and structural changes, causing irreversible cellular damage (2). The model of iso-induced myocardial ischemia is considered as one of the most widely used experimental model to study the beneficial effects of many drugs and cardiac function (3).

Morinda citrifolia (Rubiaceae) is a small tree that grows widely across Polynesia. Noni has been traditionally used for its antibacterial, antiviral, antifungal, antitumor, antihelmintic, analgesic, hypotensive, anti-inflammatory, and immune-enhancing effects. Furthermore, noni juice is currently widely consumed for its purported ability to prevent various diseases such as diabetes, hypertension, cancer, cardiomyopathy, and cerebral apoplexy caused by arteriosclerosis (4). Kamiya et al. (5) studied the effects of the fruits of noni in preventing arteriosclerosis. During our research for natural products capable of preventing various diseases, we investigated the ability of the chemical constituents of noni in improving myocardial infarction.

Aspirin is the first nonsteroidal anti-inflammatory (NSAID) drug and has been in use for its anti-inflammatory, antipyretic, and analgesic properties. More recently, low-dose aspirin is being evaluated for the prevention of two leading causes of death, cardiovascular diseases (including stroke, myocardial infarction, and thromboembolism) and cancer (6, 7).

Iso is also well-known to generate free radicals and to stimulate lipid peroxidation, which may be a causative factor for irreversible damage to the myocardial membrane (3). Increase in oxidative stress with iso treatment causes...
oxidation of lipids, proteins, and DNA in the myocardium causing alteration in the cell structure and function (8-11). SOD activity significantly decreased with iso due to an excessive formation of superoxide anions. A decrease in SOD activity may result in the decreased removal of superoxide anions, causing free radical-induced myocardial damage (12, 13).

Apoptosis or programmed cell death is a highly regulated and energy-requiring process. The fact that apoptosis plays a role in the tissue damage after myocardial infarction (14). Iso also increases lysosomal enzyme activities. Iso-induced myocardial infarction shows many metabolic and morphological aberrations in the cardiac tissues of experimental animals similar to those observed in human myocardial infarction (2). Caspases are the most prominent group of proteases involved in the regulation and execution of apoptosis. The term “cathepsin” stands for “lysosomal proteolytic enzyme” regardless of the enzyme class. Sequence homologies of the cysteine proteases cathepsins B, C, H, L, and S indicate that these enzymes diverged early during eukaryotic evolution (15).

We studied the cardioprotective, antiapoptotic, and antioxidant effects of noni and low-dose aspirin against iso-induced myocardial ischemia. This prompted our research on the protective effects of noni and low-dose aspirin in iso-induced myocardial ischemia.

**MATERIALS AND METHODS**

**Test animals:** Forty adult male Wistar rats weighing 150-250 g were obtained from TICAM (Medical and Surgical Experimental Research Centre, Eskisehir, Turkey) and housed in polycarbonate cages in a room with controlled temperature (22°C±2°C) and humidity (50%±5%) and a 12-h cycle of light and dark (lights on from 7 a.m. to 7 p.m.). All animal procedures were approved by the Ethical Committee of Eskisehir Osmangazi University Rats were separated into five different groups (n=8, each group, respectively): control (untreated) and iso [untreated and subcutaneously injected with iso 100 mg/kg for 2 consecutive days] and three treatment groups, iso+aspirin (30 mg/kg/day for 7 days followed by 100 mg/kg iso at the end of seventh and eighth days), iso+noni (20% v/v/day nioni for 7 days followed by 100 mg/kg iso the end of seventh and eighth days), and iso+aspirin+noni (30 mg/kg/day aspirin and 20% v/v day noni for 7 days followed by 100 mg/ kg iso at the end of seventh and eighth days). At the end of the experimental period, 6 h later, the animals were sacrificed under ketamine-xylazine anesthesia. Blood samples were taken intracardially to measure serum CK-MB levels. Following the experiment, parts of the left ventricle were kept at −80°C to measure the levels of lysosomal and cytosolic cathepsins B and L ratios, caspase-3 activity, DNA fragmentation, SOD, catalase, and MDA levels.

**Experimental design:** Myocardial infarction was induced by subcutaneously injecting iso (100 mg/kg) for two consecutive days (2). 20% v/v/day noni was given to the noni groups of rats with drinking water for 7 days (16). 30 mg/kg/day aspirin was dissolved in drinking water and given to all aspirin groups of rats by oral gavage for 7 days (17).

**Caspase-3-like enzyme activity:** Caspase-3-like enzyme activity was measured using the Sigma colorimetric kit. (Sigma CASP-3-C) Frozen cardiac tissue was homogenized in a chilled lysis buffer provided in the kit, incubated over ice for 15 min and centrifuged at 3,000 rpm at 4°C for 20 min to remove the cellular debris. The supernatants were recentrifuged twice at 14,000 rpm at 4°C for 15 min each. The supernatants were used to form samples. For the caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Val-Asp p-nitroanilide by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety. The concentration of pNA released from the substrate was calculated from the absorbance values at 405 nm or from a calibration curve prepared using defined pNA solutions. Caspase-3 enzyme activity was calculated to receive µmol of pNA released per min per mg of protein (20).

**DNA fragmentation assay:** Frozen cardiac tissues were homogenized in lysis buffer 1:10 w/v; (5 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). The homogenate was then centrifuged at 27000g for 20 min to separate the intact chromatin in the pellet from the fragmented DNA in the supernatants. Pellets were obtained from TICAM (Medical and Surgical Experimental Research Centre, Eskisehir, Turkey) and housed in polycarbonate cages in a room with controlled temperature (22°C±2°C) and humidity (50%±5%) and a 12-h cycle of light and dark (lights on from 7 a.m. to 7 p.m.). All animal procedures were approved by the Ethical Committee of Eskisehir Osmangazi University Rats were separated into five different groups (n=8, each group, respectively): control (untreated) and iso [untreated and subcutaneously injected with iso 100 mg/kg for 2 consecutive days] and three treatment groups, iso+aspirin (30 mg/kg/day for 7 days followed by 100 mg/kg iso at the end of seventh and eighth days), iso+noni (20% v/v/day nioni for 7 days followed by 100 mg/kg iso the end of seventh and eighth days), and iso+aspirin+noni (30 mg/kg/day aspirin and 20% v/v day noni for 7 days followed by 100 mg/ kg iso at the end of seventh and eighth days). At the end of the experimental period, 6 h later, the animals were sacrificed under ketamine-xylazine anesthesia. Blood samples were taken intracardially to measure serum CK-MB levels. Following the experiment, parts of the left ventricle were kept at −80°C to measure the levels of lysosomal and cytosolic cathepsins B and L ratios, caspase-3 activity, DNA fragmentation, SOD, catalase, and MDA levels.

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were resuspended in 0.5 N of perchloric acid, and the concentrated perchloric acid was added to the supernatant samples to a final concentration of 0.5 N. Samples were heated at 90°C for 15 min and centrifuged at 1500´g for 10 min to remove protein. If the color reactions were available for the determination and identification of DNA, samples would react with diphenylamine for 16 to 20 h at room temperature. Absorbance was measured spectrophotometrically at 600 nm. Results were expressed as percentages of supernatant/pellet (21).

**Protein determination of cardiac tissues**: Protein levels in the heart were determined using the biuret method (22).

**Malondialdehyde levels**: Cardiac tissues were homogenized in 0.15 N KCl. Homogenates were centrifuged at 3000’g for 10 min. The supernatants were used to measure MDA levels by the colorimetric reaction of thiobarbituric acid reactive substances in the presence of MDA at 532 nm, according to the method described by Ohkawa et al. (23). The results were expressed as nmol/mg protein.

**SOD and catalase enzyme activities**: SOD activity was measured on the basis of the ability of the enzyme to inhibit the reduction of nitroblue tetrazolium by a superoxide radical, which was generated by the photoreduction of riboflavin and oxygen, according to method described by Winterborn et al. (24). The decomposition rate of hydrogen peroxide by CAT was used to assay the enzyme activity, according to the method described by Beutler (25). SOD and CAT activities were expressed as units per milligram of protein (U/mg protein).

**Statistical analysis**

The data were presented as mean±SEM and analyzed using Statistical Packages for the Social Sciences (SPSS) for windows version 15.0 (SPSS Inc.; Chicago, IL, USA) and Sigma stat 3.5. If data passed the normality test, the significance of differences among the groups was determined using the Tukey’s test, after significant one-way ANOVA. On the other hand, if data did not show normal distribution among the groups, Kruskal-Wallis test was used to compare parameters. p<0.05 was considered statistically significant.

**RESULTS**

Figure 1 shows the serum CK-MB activity in all groups. The serum CK-MB activity was elevated significantly in the iso, iso+noni, and iso+aspirin groups compared with the control group (p<0.05). Also, this activity was found to be lower in the iso+noni, iso+aspirin, and iso+noni+aspirin groups than in the iso group, but it was statistically insignificant (p>0.05).

Figure 2 shows the levels of DNA fragmentation, cathepsin B C/L ratio, cathepsin L C/L ratio, and caspase-3 activity in the cardiac tissues of control and experimental rats. DNA fragmentation was increased in the iso group compared with the control group (p<0.05). In addition, DNA fragmentation was decreased in the iso+noni, iso+aspirin, and iso+noni+aspirin groups compared with the iso group (p<0.05, p<0.01, and p<0.01, respectively). Cathepsin B C/L ratio was statistically significant only in the iso+noni group (p<0.001). Also, cathepsin L C/L ratio was decreased in the iso+noni+aspirin groups compared with the iso group, but it was statistically significant only in the iso+noni group (p<0.001). Also, cathepsin L C/L ratio in the iso+noni+aspirin group was higher than that in the iso+noni group (p<0.05). Caspase-3 activities in the iso group were higher than those in the control group (p<0.01). Caspase enzyme activities were decreased in all treatment groups compared with the iso group, but these activities were statistically significant only in the iso+noni group (p<0.05).

**DISCUSSION**

The significant increase in the levels of cardiac markers in iso-induced rats is an indication of the severity of necrotic damage in the myocardium induced by iso. There was a significant elevation in serum CK-MB confirming the acute myocardial infarction in rats. These observations were in line with previous studies conducted on rats treated with iso (26). CK-MB is localized predominantly in the heart, and this makes it a valuable diagnostic marker for MI, as damages specific to the myocardium would result in the elevation of serum CK-MB levels (10, 11, 27). Our results show that the serum CK-MB activity was significantly elevated in the iso, iso+noni,
and iso+aspirin groups compared with the control group. Also, this activity was found to be lower in the iso+noni, iso+aspirin, and iso+noni+aspirin groups than in the iso group, but this was not statistically significant.

An increase in oxidative stress was recorded during and following myocardial infarction by many researchers (28-30). The metabolic products of iso are responsible for the production of free radical and myocardial damage in iso-induced rats. Li et al. (31) observed decrease in the activities of SOD and catalase in iso-induced rats. The free radicals utilized the endogenous antioxidant enzymes SOD and catalase and made the myocardium more susceptible for further free radical attack (31). The iso alone group showed a significant decrease in myocardial endogenous antioxidant SOD compared with the control and nonmyocardial infarction groups. A decrease in SOD activity may result in the decreased removal of superoxide anions, which can cause free radical-induced myocardial damage (32, 33). Our findings show that noni and aspirin reduced oxidative biomarkers against iso-induced myocardial infarction. We think it is due to antioxidant properties of noni and aspirin in cardiac tissues.
Aspirin treatment is found to counteract the effect of iso on lipid and lipid peroxide formation and associated enzyme changes in heart mitochondria (34). Low-dose aspirin has become standard therapy in primary and secondary prophylaxis of myocardial infarction (35).

Noni has been reported to possess antithrombotic, antioxidant, analgesic, anti-inflammatory, and xanthine oxidase inhibitory activities (36, 37). There are also preliminary studies reporting its blood pressure lowering and vasodilatory properties. However, the possible models of actions of cardiovascular activities are lacking (38). It is known that oxidative stresses, such as generation of damaging reactive oxygen species, will lead to cell death under ischemic condition. In addition, it is reported that ischemic neuronal damage could be restored by vitamins and polyphenols, both of which have antioxidative properties (39).

Iso-induced rats showed increased DNA fragmentation, caspase-3, cytochrome c, and tumor necrosis factor-α (TNF-α) (12). The activities of lysosomal enzymes (β-glucuronidase, β-N-acetyl-glucosaminidase, β-galactosidase, cathepsin B, and cathepsin D) were increased significantly in serum and the heart of iso-induced myocardial-infarcted rats (40, 41). Myocardial enzymes, cytokines, oxidative stress, blood coagulation times, a marker for early-stage apoptosis, caspase-3 activity, and expression levels of BAX, BCL-2, and FAS in isolated primary cardiomyocytes were examined. In contrast, with the control and sham groups, significant increases in the following parameters were measured in the blood of acute myocardial infarction group animals (42). The current study measured the levels of DNA fragmentation, cathepsin B C/L ratio, cathepsin L C/L ratio and activity of caspase in the heart tissue of all groups. DNA fragmentation was increased in the iso group compared with the control group. DNA fragmentation was decreased in the iso+noni, iso+aspirin, and iso+noni+aspirin groups compared with the iso group. Cathepsin B C/L ratio was significantly elevated in the iso group compared with the control group. This ratio was decreased in the iso+noni, iso+aspirin, and iso+noni+aspirin treatment groups compared with the iso group, but these levels were statistically significant only in the iso+noni+aspirin group. Cathepsin L C/L ratio was increased in the iso group compared with the control group. Cathepsin L C/L ratio was decreased in the iso+noni and iso+noni+aspirin groups compared with the iso group, but statistically significant only in the iso+noni group. Also, cathepsin L C/L ratio was higher in the iso+noni+aspirin group than in the iso+noni group. Caspase-3 activities were higher in the iso group than in the control group. Caspase enzyme activities were decreased in all treatment groups compared with the iso group, but these activities were statistically significant only in the iso+noni group. Our results showed that noni with apoptotic parameters alone may reduce apoptosis.

In fact, this is the first study reporting the antioxidant, antiapoptotic, and cardioprotective effects of noni and low-dose aspirin in iso-induced myocardial ischemia in rats. Generally, our results demonstrated that noni and noni+aspirin were decreased apoptotic markers.

**CONCLUSION**

Our results demonstrate that noni and low-dose aspirin have the ability to protect cardiac tissue ischemic injury through antiapoptotic and antioxidant effects that provides a basis for further study and the development of noni and low-dose aspirin as a promising agent for treating coronary heart disease.

**Ethics Committee Approval:** Ethics committee approval was received for this study from the ethics committee of Eskişehir Osmangazi University (24-143).

**Informed Consent:** The informed consent was not required because the study was performed on animals.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Conceived and designed the experiments or case: KKO., GK., MEI. Performed the experiments or case: KKO., AOL, KK. Analyzed the data: KKO., GK., AOL. Wrote the paper: KKO. All authors have read and approved the final manuscript.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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