Cardioprotective role of long-term kefir and omega-3 fatty acid supplementation on myocardial apoptosis via oxidative stress-mediated lysosomal cathepsin release in isoproterenol-induced myocardial infarction rat model

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ABSTRACT

Objective: The antiapoptotic and antioxidative role of long-term kefir and omega-3 fatty acids and their relationship with cysteine proteases on isoproterenol (ISO) induced myocardial infarction (MI) experimental model was investigated in our study.

Material and Methods: Fifty male Sprague-Dawley rats were evenly divided into five distinct groups (n=10): Control, MI, kefir +MI, omega-3+MI, and kefir+omega 3+MI groups. Kefir 10% (with drinking water) and omega-3 fatty acid (30 mg/day per 100g body weight into the standard chow) were administrated during 30 days. ISO was subcutaneously injected into the rats (100 mg/kg b.w.) on the 29th and 30th days. Myocardial tissue and blood samples were taken 12 hours after the last ISO dose. Creatine kinase MB (CK-MB) activities were measured in serum samples. Caspase 3, superoxide dismutase (SOD), malondialdehyde (MDA), nitric oxide (NO), DNA fragmentation, cathepsin B and L levels, were measured in myocardial tissue.

Results: Serum CK-MB (p<0.05) and cardiac tissue MDA (p>0.05), NO (p<0.01), caspase 3 (p<0.01), DNA fragmentation (p<0.001), cathepsin B (p<0.05) and L (p<0.05) activities were increased and SOD (p<0.001) activities were decreased in MI group compared to control group. The preventive effects of long-term therapy with kefir and omega-3 fatty acids have been demonstrated on apoptosis, oxidative stress markers, and cysteine protease enzymes.

Conclusion: Our results showed that long-term administration of kefir and omega-3 fatty acids might be effective in reducing myocardial apoptosis through oxidative stress-mediated release of cysteine proteases in myocardial infarction, especially in the kefir and combined therapy groups.

Keywords: Apoptosis, Cathepsin, Kefir, Myocardial infarction, Omega-3 fatty acids

INTRODUCTION

Myocardial infarction (MI) is one of the most common causes of death worldwide. Many molecular changes occur in acute MI (1). The most important molecular alteration in this process begins with cardiac triggering, which leads to ventricular remodelling characterized by cardiac dilatation, decreased contractile power, and consequently, a progressive decline in cardiac function. Apoptosis and necrosis are key molecular events in this progression (2). Apoptosis plays a vital role in the myocardial loss after acute MI. Although cardiomyocyte death in the acute phase of infarction occurs via both necrosis and apoptosis, in the chronic phase of infarction, only apoptotic cell death is observed (3).

In the early period of acute myocardial infarction (AMI), myocardial apoptosis was found to peak between 6-12 hours and lasts until day 10 (4). In addition, apoptosis has been described in the sub-acute and late period of MI. According to some researchers, apoptosis is the primary mechanism of cell death in AMI and is suggested to be more responsible in tissue loss than necrosis (5).
Many factors, such as reactive oxygen derivatives, hypoxia, calcium overload and neurohormones trigger myocardial apoptosis. These factors activate caspases, which are the basis for apoptotic events with a number of genetic changes. Caspases cause cell death by causing destruction of target nuclei in the nucleus, cytosol and mitochondria. Lysozyme cysteine proteases, also known as cathepsins, are involved in the initiation of apoptosis, similar to caspases. Cathepsin B and L are members of a protease, with cathepsin L acting as an indirect initiator of pro-caspase 3 activation through another lysosomal membrane-bound protease. Lysozyme proteases trigger apoptosis by acting on mitochondria, generating mitochondrial dysfunction associated with the release of pro-apoptotic factors (7). It has been demonstrated that the production of oxidative stress may lead to lysosomal membrane permeabilization and apoptosis through cathepsin enzymes into cytosol (8). Recent studies showed the relationship between oxidative stress and cardiovascular diseases such as MI, ischemia and atherosclerosis. Reactive oxygen species (ROS) cause deleterious effects by affecting the cellular components such as lipid, DNA, protein and carbohydrates (9). The most important product of lipid peroxidation is malondialdehyde (MDA). Too much production of ROS can cause excessive peroxidation of membrane lipids, leading to disruption of the lysosomal membrane. Induction of lysosomal membrane permeabilization by lysosomal membrane peroxidation reactions has been extensively studied in model systems, and it is hypothesized that intralysosomal iron, react with hydrogen peroxide, and generates highly reactive hydroxyl radicals that initiate peroxidation of the lysosomal membrane (10). ROS are eliminated by enzymatic and non-enzymatic antioxidant defense systems like superoxide dismutase (SOD) (11). In vitro and in vivo studies in cardiomyocytes, and the myocardium have shown that peroxynitrite (ONOO⁻), which is formed from nitric oxide, triggers apoptosis (12).

Kefir is a fermented milk drink with a slightly sour taste creamy consistency and is a probiotic. Kefir is separated from other fermented dairy products by the production of kefir grains, which are specialized and biological living organisms. These grains grow, multiply and transfer their properties to the next generation. Previous studies demonstrated that kefir has antiapoptotic and antioxidative properties (13, 14). Omega-3 (n-3) fatty acids are the precursors of Eicosapentanoic acid (EPA). Omega-3 fatty acids have many health benefits, which protects cardiovascular health, reduces triglyceride levels, increases HDL known as good cholesterol, helps reduce the risk of heart attack (15). Studies have shown that omega-3 exhibits antiapoptotic and antioxidative properties (16,17).

In this study, we aimed to investigate the cardioprotective effects of long-term omega-3 fatty acid and kefir supplementation on myocardial apoptotic cell death, dependent on oxidative stress mechanisms, in an ISO-induced myocardial infarction (MI) model. We also investigated the possible triggering role of lysosomal release of cathepsins on myocardial apoptotic cell death. For this purpose, in order to analyze apoptosis and oxidative stress; Caspase-3, DNA fragmentation, Lysosomal Cathepsin B and L, MDA, NO and SOD were measured in myocardial tissue of ISO-induced rats.

MATERIALS and METHODS

Fifty male Sprague-Dawley rats (150-200g) were used in this study. Animals were kept at a controlled temperature (22±2°C) and 12-hour light and dark cycle and were randomized into 5 equal groups (n=10).

1. Control group: The rats in this group were given standard diet and water for 30 days.

2. MI group: Rats were fed standard diet and water for 30 days. MI was induced with ISO on the 29th and 30th days.

3. Kefir + MI group: Kefir 10% was supplemented with drinking water for 30 days, and MI was induced.

4. Omega-3+MI group: Rats were fed standard diet supplemented with Omega-3 (30 mg/day per 100g body weight (b.w)) and MI was induced.

5. Kefir + Omega 3 + MI group: Rats were fed a standard diet supplemented with Omega-3 (30 mg/day per 100g b.w.) and had free access to water supplemented with 10% kefir for 30 days, and then MI was induced on day 29 and 30.

Preparation of Kefir and Omega-3: Kefir 10% was added to the drinking water for 30 days, taken from the Ankara University Department of Milk and Milk Products Technology. Kefir yeast (4 g) was added to 500 mL milk and incubated for 24 hours at room temperature to be fermented. At the end of this period, the gel mixture was passed through a filter, and aqueous part was used (18). Omega-3 (30 mg/day per 100g b.w.) was added to the standard chow of the animals for 30 days (19). Chows were rebuilt every two days.

Induction of MI: ISO (Sigma Aldrich, I6504) was dissolved in physiological saline and was subcutaneously injected into rats (100 mg/kg b.w.) for 2 days with 24-h intervals to induce experimental MI (on the 29 and 30th days). Blood and tissue samples were taken 12 hours after the last ISO dose was applied (20).

The rats were euthanized under a high dose of anesthesia (Ketamine/Xyazine 3:1 intraperitoneal), and blood samples were obtained by cardiac puncture. Heart tissue was dissected and stored at -80°C until the biochemical analyses.

Biochemical Analysis

Serum CK-MB Assay: CK-MB activities were measured in serum samples using the Roche/Hitachi Modular P analyzer. CK-MB levels were expressed as U/L.

For the measurement of MDA levels, tissue samples were homogenized in 0.15 N KCl solution. The homogenized tissue suspension was centrifuged at 3000xg for 10 minutes. MDA levels were determined using the method described by Ohkawa et al. in 1979 and the results were reported as nmol/mg protein (21).

SOD activities were measured at 560 nm, and the results were reported as IU/mg protein (22).

NO levels were measured using Cortas and Wikid's method. NO levels were reported as μmol per mg of protein (23).
Determination of Cathepsin B and L enzyme activity was performed by obtaining lysosomes (FEL) and supernatant (cytosolic) fractions using the method modified by Ichihara et al. Tissues were lysed in sucrose Tris-HCl buffer (pH 7.4) and then centrifuged at 1000×g for 5 min at +4 °C. The pellets were removed and stored at −80 °C. Supernatant fractions were centrifuged at 10 000 ×g for 20 min at +4 °C. The collected FEL fractions were maintained on ice for 1 hour. Measurements were made by measuring fluorescence intensities at 348 nm excitation and 440 nm emission for 5 min. Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride substrate for cathepsin B and Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride substrate for cathepsin L were used. Results are reported as units/mg protein. Measurements were taken in cytosolic (C) and lysosomal (L) fractions, and the results were reported as C/L. C/L ratio indicates the extent of lysosomal integrity (24,25).

Caspase 3 activities were measured with the kit (Sigma-APC165). Results were reported μmol of pNA released per min per mg of protein (26).

DNA fragmentation was measured using Atroshi’s method. Results were reported as percentages of supernatant to pellet (27).

Tissue protein levels were determined using the Bradford method. Results were reported as mg/ml (28).

Statistical Analysis: Statistical data analysis was performed using SPSS software, version 21.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Tukey’s multiple comparison test were used. Results were presented as mean ± standard deviation, and p<0.05 was considered statistically significant.

RESULTS

Serum CK-MB activities are shown in Figure 1. CK-MB in the MI, kefir+MI, omega-3+MI group and kefir+omega-3+MI groups were significantly increased compared to the control group (p<0.05, p<0.05, p<0.01, p<0.05). On the other hand, CK-MB levels in kefir+MI and kefir+omega-3+MI groups demonstrated a reduction compared to the MI group (p>0.05).

Figure 1: Effect of Kefir and/or omega-3 on serum enzymes.
a: p<0.05 compare with control group; b: p<0.01 compare with control group.

Oxidative stress parameters are shown in Figure 2. SOD activities in the MI group were lower than the control group (p<0.001) and elevated in other therapy groups as compared to MI group, and this elevation was statistically significant in the kefir+MI group (p<0.05). MDA levels were moderately high in MI group as compared to the control group (p<0.05) and also low in the kefir+MI, and kefir+omega-3+MI groups as compared to MI group, (p<0.01). NO levels were increased in the MI group were higher than in the control group (p<0.01) and significantly decreased in kefir + MI and kefir + omega-3+MI groups compared to MI group (p<0.05).

The parameters of apoptotic cell death are shown Figure 3. Caspase-3 activities in all groups were higher than in the control group, and this increase was statistically significant in the only MI group (p<0.01). DNA fragmentation was significantly higher in all groups compared to the control group. Only the MI group exhibited a statistically significant increase (p<0.001). All therapy groups decreased statistically compared to the MI group (p<0.001).
Lysosomal proteases are shown in Figure 4. The C/L ratio for cathepsin B in the MI group was higher than in the control group, but it was not statistically significant (p>0.05). This ratio was decreased in the all-treatment groups as compared to the MI group (p>0.05). C/L ratio of cathepsin L activities in MI group was higher than in the control group (p>0.05). The statistical significance of this rate was observed only in the kefir + MI and omega-3 + MI groups compared to the MI group (p<0.01 and p<0.05, respectively). At the same time, a decrease was also seen in the kefir + omega 3 + MI group (p>0.05).

DISCUSSION

ISO-induced MI, has been reported to cause biochemical and pathophysiological changes similar to those observed in human MI in the heart tissue of the experimental animals. ISO is a synthetic catecholamine and β-adrenergic agonist, which affects the myocardium through free radical production. Oxidative stress, mitochondrial dysfunction, and apoptosis are the main pathologic events that lead to myocardial damage (29).

Recent studies indicated that ISO-induced cardiomyopathy altered the cardiac marker enzymes activities such as CK-MB. Increased activities of cardiac enzymes in serum indicate cellular damage, loss of functional integrity and/or permeability of the cell membranes (30). CK-MB activities in our study were elevated in the MI group compared to the control group. In our study, we observed that CK-MB was decreased in all therapy groups. Mert et al. reported that increased cardiac enzymes due to MI were reduced by the kefir administration (31). It has been shown that omega-3 polyunsaturated fatty acids are related with lower CK-MB (32).

SOD which converts superoxide radicals into hydrogen peroxide and molecular oxygen, is a protective enzyme against oxidative stress (33). In our study, we found that SOD activities were decreased in MI group. Decreased SOD activities can lead to the formation of excessive superoxide anions, which may cause oxidative stress. SOD activities were increased in kefir and/or omega-3 fatty acid groups, especially kefir+MI group, as compared to MI group. Kefir, known for its probiotic properties, has been associated with a decrease in ROS production and an increase in SOD activities (34).
We think that Kefir and omega-3 administration have antioxidative effects through regulating SOD activity. MDA, which ends product of the lipid peroxidation pathway, is an important peroxidative injury marker. ISO-injection causes increased oxidative stress markers in rats depending on ROS production from auto-oxidation of ISO (35). In our study, peroxidative injuries were decreased in kefir and omega-3 combined therapy groups according to MI group. Kefir and omega-3 were previously shown to reduce the level of MDA by inhibiting lipid peroxidation under various pathological conditions (36).

Previous studies have shown that omega-3 fatty acids protect against oxidative damage. Kus et al. also found that SOD activities were increased, and MDA levels were decreased in rats given omega-3 fatty acids (37). In our study, cardiac tissue NO levels were significantly higher in the MI group compared to the control group. High levels of tissue NO have supported the notion that NO plays a role in the pathogenesis of ISO-induced myocardial oxidative injury. Depre et al. found an increase in NO accumulation and myocardial damage due to an increase in NO synthase activity after MI in rabbit hearts (38). In our study, NO levels in cardiac tissue were found to be lower in all the treatment groups than in the MI group. These reductions were statistically significant in the kefir+MI and kefir+omega-3 groups when compared to the MI group. We think that long-term administration of kefir and omega 3 have antioxidative properties in myocardial injury via regulating SOD, MDA and NO.

Apoptosis is related to myocardial injury in acute MI, which participates in subsequent left ventricular remodeling and symptomatic heart failure. Caspase 3 and DNA fragmentation are intrinsic apoptotic biomarkers (39). In our study, we found increased caspase-3 activities and DNA fragmentation in rats’ ISO-induced MI, which might be due to apoptotic pathways. In kefir, omega-3 and combined therapy groups, caspase-3 and DNA fragmentation were lower than in the MI group. In our previous study, parallel to this study, we found that caspase-3 and DNA fragmentation were increased in ISO-induced MI (20). Matsu et al. demonstrated that administration of kefir has been shown to reduce apoptosis and caspase-3 activation. Antiapoptotic effect of kefir is related to the inhibition of caspase-3 (14). Also, it has been reported that kefir supplementation has a protective role on ultraviolet-induced apoptosis in melanoma cells (40). In another study, it was found that probiotics had a protective effect against liver ischemia-reperfusion injury by increasing superoxide dismutase enzyme activity (41). It has also been found that Kefir increases levels of some antioxidants, such as GSH, in rats that had abnormal formation of colony crypts induced by azoxymethane administration, and it was stated that Kefir plays an antioxidant role (42). Chen et al., demonstrated that omega-3 polyunsaturated fatty acid attenuates apoptotic cell death in traumatic brain injury (43). Another study showed that omega-3 fatty acids protect against cardiomyocyte apoptosis induced by hypoxia (44).

Increased cathepsin L levels are related to coronary artery stenosis and coronary heart disease. Inhibition of myocardial cathepsin L release during reperfusion following MI protects cardiac functions and reduces infarct size (40). Liu et al., reported that cathepsin B inhibition with CA-074Me prevents cardiac dysfunction and remodeling following myocardial infarction in experimental model (45). Lysosomal enzymes, especially cathepsin L, were decreased in the therapy groups compared to the ISO-induced myocardial infarction group. It has been demonstrated that lysosomal cathepsins trigger apoptosis (7,8). In our previous study, which was parallel to this study, we found that cathepsin B and L levels were increased in ISO-induced MI (20). In another previous study, we demonstrated that inhibition of cathepsin B and L release and nitric oxide production related to protection of caspase-3 mediated neuronal apoptosis (46).

CONCLUSION

Our data indicate that myocardial apoptosis and oxidative stress injury are present in MI. We hypothesize that apoptotic cell death is linked to the release of lysosomal cysteine protease enzymes into the cytosol, driven by increased oxidative stress. Moreover, long-term therapy with Kefir and/or omega-3 fatty acids may effectively mitigate myocardial apoptosis and oxidative stress by stabilizing lysosomal membranes and preventing the release of lysosomal proteases. Thus, inhibiting the release of proteolytic enzymes or utilizing cysteine protease inhibitors could be a promising therapeutic approach to reduce myocardial injury induced by MI.

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Authors’ Contribution

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Formal analysis: GÜŞ, AOİ, GK
Funding acquisition: -
Investigation: GÜŞ, AOİ, GK, MEİ
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Software: SPSS
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REFERENCES


