Effect of Fish and Flaxseed Oil Supplementation on Isoprenaline-Induced Myocardial Infarction in Rats: Inhibition of Mitochondrial Permeability Transition Pore Opening

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Abstract

Objectives: Dietary n-3 polyunsaturated fatty acids have positive effects on the heart. The present study investigated the effects of pretreatment with fish oil (FO) and flaxseed oil (FLO) on the heart of the rat, which is associated with the isoprenaline (ISO)-induced myocardial injury.

Materials and Methods: The study was conducted on 40 male Wistar rats which were included in control, ISO, FO + ISO, and FLO + ISO groups (each containing 10 rats). In ISO rats, acute myocardial ischemia was induced by ISO while in FO + ISO group, the rats were pretreated with FO orally for 4 weeks. Finally, rats in the FLO + ISO group received pretreatment with FO and flaxseed oil orally for 4 weeks. Eventually, the histopathological examinations of the cardiac tissues and serum activity of creatine kinase-MB (CK-MB) were assessed. Moreover, mitochondria were isolated to examine the mitochondrial swelling.

Results: Based on the results, ISO administration significantly increased the serum CK-MB activity compared to the control group. In addition, severe muscular damage to the heart was observed in more than 70% of the rats in ISO group. However, a remarkable decrease in the intensity of heart tissue destruction, as well as the serum levels of CKMB was found in the FO + ISO group compared to the ISO group. Conversely, there was no significant decrease in the serum level of CKMB in FLO + ISO group compared to the ISO group.

Conclusions: In general, pretreatment with FLO significantly suppressed the intensity of heart tissue destruction compared to the myocardial ischemic group. FO and FLO led to a decrease in CaCl2-induced swelling in the mitochondria. Therefore, FO and FLO result in protecting against ischemia/reperfusion injury through inhibiting the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

Keywords: Isoprenaline, Myocardial Infarction, Fish oil, Flaxseed oil, Cardioprotection

Introduction

Nowadays, using the omega-3 polyunsaturated fatty acids (ω3-PUFAs) extracted from fish oil (FO) has increased due to its beneficial effects on the heart (1,2). Myocardial infarction (MI) is still considered one of the most serious health problems worldwide (3), in which an imbalance between the myocardial oxygen supply and demand leads to myocardial necrosis (4). Accordingly, dietary interventions have been recently used as an effective strategy for preventing the mitigation of the risk factors for several diseases (5). Given an increase in cardiovascular diseases in recent years, researchers have focused on investigating the cardioprotective effects of

FOs, individual n-3 PUFA (6,7).

Some experimental evidence suggests that regular consumption of (n-3) PUFA has remarkable protecting effects against myocardial ischemia (8). Experimental and human intervention studies reported an inverse association between the dietary intake of FO or fish and mortality caused by cardiovascular diseases (7,9). The results of animal studies investigating the effects of FOs showed that these oils have antiarrhythmic effects and can result in improving the heart function in the early post-ischemic situation and protecting against ischemia-reperfusion injury (9,10). In addition, such oils are called essential fatty acids since they are needed; further, PUFAs

such as linoleic acid and alpha-linolenic acid (ALA) cannot be synthesized in the human body. Furthermore, based on the reports, FOs (e.g., Eicosapentaenoic and Docosahexaenoic acids) and some plant oils such as flaxseed oils (FLOs) are regarded as the most common sources of n-3 PUFA (11).

Indeed, although the role of long-chain PUFAs in preventing the lipid synthesis and promoting the B-oxidation of fatty acids is well-recognized, the functional mechanisms of such fatty acids are not well-discovered. Moreover, various studies investigated the effects of some medicinal plants and marine n-3 PUFA on the myocardial ischemia; however, they reported conflicting results regarding preventing congenital heart disease complications (12,13). Additionally, it is still undetermined how dietary fatty acid can prevent the myocardium from ischemia-reperfusion injury. Isoprenaline (ISO) (i.e., a b-adrenoceptor agonist) can lead to an induction of MI in high doses (14), therefore, the present study sought to make MI model in the rats. In addition, the present study attempted to compare the effects of pretreatment with FO or FLO on ISO-induced MI in rats.

Materials and Methods
Chemicals and Reagents
FLO was purchased from the Barij Essence Pharmaceutical Company (Iran). This oil contained 7700 mg ALA (Omega-3), 2170 mg linoleic acid (omega-6), and 2240 mg of oleic acid (omega-9). Further, the FO and isoproterenol were purchased from Sigma-Aldrich Company.

Animals and Experimental Groups
In this experimental study, 40 Wistar rats (weighing 250-300 g) were used. Based on the guidelines for maintaining the animals from the Research and Technology Deputy of Gonabad University of Medical Sciences, the rats were housed in the standard situation including a room temperature of 23±2°C, the humidity of 60%-70%, and a 12/12-hour light/dark cycle with free access to food and water. Furthermore, 100 mg/kg ISO was first dissolved in one ml of normal saline in order to induce the experimental myocardial injury model and then, the solution was subcutaneously (S.C.) given to the rats once a day for 2 consecutive days (15). Next, the rats were randomly divided into 4 groups (n = 10/each group) as follows.

Group 1: The control group in which the rats were orally treated with saline (1 cc/kg) for 4 weeks, then, received saline for 2 successive days by subcutaneous injection;

Group 2: The experimental group 1 (ISO) in which the rats received 1 cc/kg of saline (similar to the control group) and treated with ISO subcutaneously (100 mg/kg) for 2 consecutive days;

Group 3: The experimental group 2 (FLO + ISO) in which the rats first received FLO (0.4 g/kg) orally for 4 weeks, then myocardial ischemia was induced by using

the ISO;

Group 4: The experimental group 3 (FO + ISO) in which the rats received FO (0.4 g/kg) orally for 4 weeks and myocardial ischemia was induced similar to the other experimental groups. Finally, all the rats were anesthetized with ketamine/xylazine (60 mg/kg + 5 mg/kg, i.p.). Then, their blood samples were collected for separating the serum, as well as estimating the lactate dehydrogenase (LDH) and creatine kinase-MB (CKMB) activity. Moreover, after sacrificing, their hearts were rapidly isolated, washed with ice-cold saline, and cut into two equal halves. One half was homogenized in phosphate buffer (pH 7.4) to prepare 10% (w/v) homogenate. This solution was used for isolating the mitochondria. Additionally, the other half was immersed in 10% formalin for histopathological examinations in the cardiac apex of the myocardium employing hematoxylin-eosin (H & E) staining.

Biochemical Assays
The activity of CKMB and LDH were analyzed using the commercial kits (Pars Azmoon, Iran) and an autoanalyzer (Roche Hitachi, Germany).

Histopathological Assays
Samples were routinely fixed in 10% buffered formalin (Sigma-Aldrich, Germany), embedded in paraffin, and stained after the sections (5 μM thick/each section) by H & E. Then, lesions in the samples were graded as follows.
- Grade I or nil (score=0);
- Grade II or minimum (score=1) when the focal myocyte damage was observed;
- Grade III or mild (score=2) when a slight degree of inflammatory process was found in addition to the focal myocyte damage;
- Grade IV or moderate (score=3) when the diffuse inflammatory process was detected, along with extensive myofibrillar degeneration;
- Grade V or severe (score=4) when the diffuse inflammatory process was observed, along with necrosis (16).

Mitochondrial Isolation From the Heart Tissue
First, the cardiac tissue was minced and washed using the phosphate-buffered saline (PBS, Sigma-Aldrich), then suspended into 10 ML of mild trypsin digestion at 4°C for 30 minutes. In addition, the solution was diluted by the medium containing bovine serum albumin and trypsin inhibitor. Next, the solution was centrifuged at 600 g for 10 minutes to obtain the mitochondrial pellet, then the supernatant was separated and centrifuged at 8000 g for 15 minutes. Afterward, the obtained upper layer was discarded while the lower dark layer was separated and washed. Then, the isolated mitochondria were purified on a discontinuous gradient including 6% Percol l, as well as 17% and 35% metrizamide. Eventually, the solution was centrifuged at 5000 g for 30 minutes and a narrow band of

mitochondrial sediment was recovered (17). Moreover, the total protein concentration of the isolated mitochondrial was determined by the Bradford method (18).

Mitochondrial Swelling Assays
The mitochondrial permeability transition pore (MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) can induce mitochondrial swelling and change the mitochondrial volume. Briefly, the isolated mitochondria (600 mg) was dissolved in one mL of respiration buffer, then incubated at 30˚C for 10 minutes. Then, $CaCl_2$ (200 mmol/L) was added to the solution in order to induce the MPTP opening (19,20). Finally, the absorbance was measured using a spectrophotometry device at 520 nm for 21 minutes within 3 minutes time intervals (19).

Statistical Analysis
The Kolmogorov–Smirnov test was utilized to determine the normal distribution of the obtained data. Further, the one-way ANOVA test, followed by Tukey post hoc test was used to compare the means between all groups of the study. Furthermore, the nonparametric Kruskal-Wallis and Mann-Whitney tests were performed to compare the total histopathological scores. All data were presented as mean ± standard error (SE) and $P < 0.05$ was considered statistically significant.

Results
Biochemical Parameters
Based on the results, ISO increased serum CKMB activity compared to the control group ($P < 0.05$). Moreover, pretreatment with FO reduced the elevated CKMB level compared to ISO group ($P < 0.05$) while FLO supplementation failed to decrease the serum CKMB activity compared to the ISO group ($P > 0.05$). Additionally, no significant differences were observed in the serum levels of CKMB between ISO + FO and ISO + FLO groups (Figure 1a). Furthermore, the activity of plasma LDH demonstrated no significant difference among the experimental groups (Figure 1b).

Histopathological Findings
During the histological assessment of the experimental group 1 (i.e., ISO) the heart of the rats showed multiple focal areas of myocardial cell degeneration with edema and inflammatory cells infiltration. In addition, pretreatment with FO and FLO modulated the myocardial degeneration effects of ISO. Further, the outstanding histopathological findings in experimental groups 3 and 4 were small multifocal degeneration with a slight degree of an inflammatory process in the subendocardial area without any severe tissue destruction. (Figure 2).

Mitochondrial Swelling Assays
Furthermore, the effects of FO and FLO on decreasing the A520 in isolated mitochondria were tested to
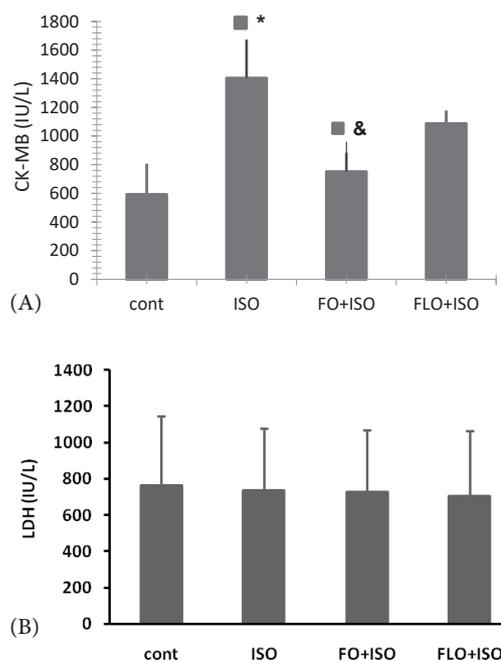
Figure 1. The Plasma Levels of CK MB **(A)** and LDH **(B)** at the End of the Experiment in Control, ISO (0.03 mg/rat), FO + ISO, FLO + ISO (0.03 mg/rat) groups. The values are expressed as mean + SEM.
Note. Cont: Control; ISO: Isoprenaline; FO: Fish oil; FLO: Flaxseed oil (gavage; 0.4 g/kg/d for 4 weeks before ischemia), CK MB: creatine kinase-MB; LDH: lactate dehydrogenase; *$P < 0.05$ vs. Cont, $^{\&}P < 0.05$ vs. ISO.
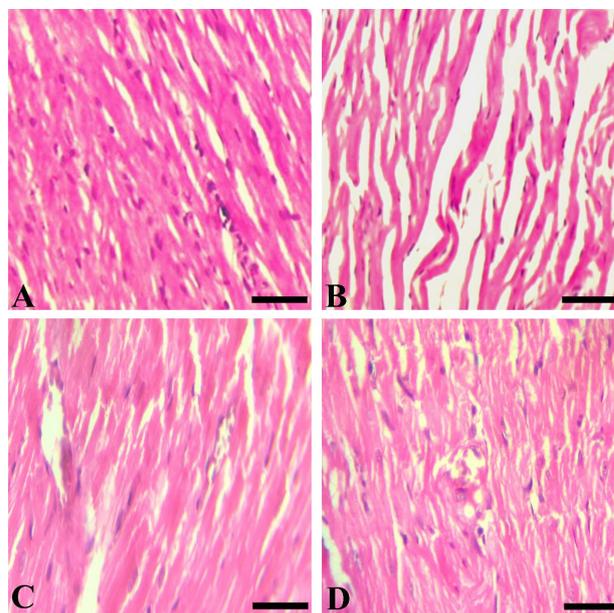

Figure 2. The Histopathological Examination of Heart in Different Groups of the Study.
A: Normal architecture of the heart tissue observed in the control group; B: Necrosis of the muscle fibers, edema, diffuse inflammatory cell infiltration, and fibroblastic proliferation found in ISO group; C: Slight degree of inflammatory process in subendocardial area, edema, and small multifocal degeneration detected in ISO+ fish oil group; D: Mild inflammatory cells infiltration and edema observed in ISO + flaxseed oil group; ISO: Isoprenaline, Scale Bar = 20 µM.

determine whether these oils can modulate the MPTP opening. Moreover, the swelling of the mitochondrion was considered the result of pore opening. Although CaCl$_2$ at the concentration of 200 mmol/L resulted in a large decrease in A520, using the FO could prevent the occurrence of this event and was confirmed by a decrease in the absorbance. Additionally, FLO in FLO + ISO group inhibited the A520 reduction (Figure 3).

Discussion

The present study evaluated the effects of fish and FLOs (i.e., FO and FLO) on MI induced by the ISO in the rats. The administration of ISO to rats caused myocardial injury, which was proved by increasing the serum CKMB level. Based on the results, treatment with FO and FLO successfully improved myocardial injury induced by the ISO. Similarly, the results of several previous studies reported that ISO leads to an increase in serum CK-MB and LDH levels in the rats (21,22). However, in the present study, although CK–MB levels increased in the serum, the serum level of LDH failed to change by administering the ISO. According to Hori et al., LDH began to rise in 24-48 hours following MI and reached a peak in 3-5 days (23). In addition, PUFAs are involved in regulating the gene expression and altering the signal transduction, cellular metabolism, and membrane lipid composition (24). On the other hand, it is reported that increasing the contractility and heart rate, hypotension, and myocardial oxygen supply/demand imbalance of the heart occur after cardiotoxicity and cardiac damages induced by ISO (25,26). Further, the induced-MI by ISO may develop due to the overproduction of free radicals resulted from the stressed condition (27). Isoproterenol can lead to myocardial damages, caused by the oxidative stress, by altering the reactive oxygen species through the auto-oxidation of the catecholamines (28-30). The findings of the present study in experimental group 1 treated with isoproterenol demonstrated severe myocardial injury and elevated serum CKMB level. There are many proposed mechanisms in order to explain the myocardial damage induced by ISO including the mitochondrial injury or dysfunction, the overload of calcium, and the generation of cytotoxic free radicals (31,32). Furthermore, based on the results of the current study, the histopathological study of the myocardial damage induced by ISO represented subendocardial necrosis, muscle fibers, and leukocytic infiltration. Similarly, Jia et al found that the consumption of oils limited the myocardial injury induced by ISO and could decrease the serum activity of CKMB. Moreover, it was confirmed that dietary fats can lead to damaging platelet and vascular function and have a role in determining the composition of fatty acids of the myocardial cell membranes (33). On the other hand, higher incorporation of DHA into the myocardial membrane phospholipid was regarded as one of the other benefits of taking the FO (34,35). Additionally, the modulation of the
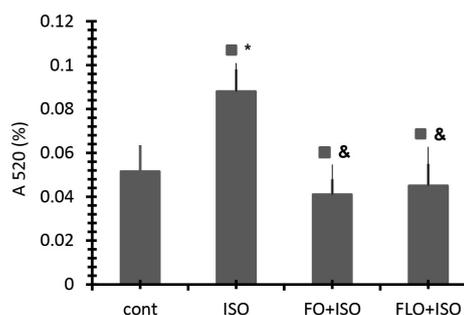
Figure 3. The Percentage of the Absorption Rate Reduction at 520 nm (A520) in All the Studied Groups. *$P<0.05$ vs. Cont, &$P<0.05$ vs. ISO.

membrane fatty acid has varied impacts on intracellular and membrane events such as intracellular lipid-based second messengers and receptor function (36,37). In the present study, daily consumption of FO could decrease the serum level of CKMB and prevent the development of myocardial injury. These events suppose that the mechanism for the cardioprotective effects of these oils is against the inotropic and chronotropic effects, which are responsible for the inhibition of cardiac calcium channels. Based on the reports, FLO had anti-inflammatory effects on healthy adult rats after the dietary intervention for 12 weeks (38,39). The results of other studies indicated that FLO has antioxidant (40) and anti-inflammatory potentials due to the antiatherogenic role of ALA (41).

Although the mechanisms of the function of ω3-PUFAs are not completely known, it can decrease arrhythmias of the heart by decreasing the blood pressure, platelet aggregation, and heart rate, as well as by ionic remodeling in the heart (42-44). In humans, improving myocardial resistance to the injuries caused by the ischemia-reperfusion is considered one of the protective impacts of the dietary n-3 PUFA against congenital heart disease complications. However, the effects of the specific dietary fatty acid on modulating the resistance of the myocardium against injuries due to ischemia-reperfusion are still unclear. Conversely, it is suggested that mitochondria can play an important role in myocardial resistance against I/R injury (45-47). In addition, according to Abdukeyum et al (12), marine n-3 PUFA could lead to a striking reduction of the infarct size (i.e., close to 80%). On the other hand, the reperfusion injury following the ischemia results in opening the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mitochondria and releasing the cytochrome C due to an increase in the cellular calcium and thus the activation of caspase cascades leading to a disruption in the generation of ATP and finally, cell death (48). Therefore, MPTP plays an important role in cardioprotection following the precondition period. The results of the current study showed that FO and FLO could protect myocyte mitochondria against I/R injury in the rat by acting on mitochondria. It was further found that the collapse of the inner mitochondrial membrane,

mitochondrial swelling, and the release of cytochrome C occur because of the opening of the MPTP (48). However, in the present study, isolated mitochondria from treatment groups exhibited reduced swelling to a challenge with 200 mmol/L $CaCl_2$.

Conclusions

In general, pretreatment with fish and FLOs could provide cardioprotection against the injuries caused by the induced-MI through ISO in rats by inhibiting the mitochondrial permeability transition pore opening in the heart of the rat.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

All the experiments were performed in accordance with the institutional guidelines of the Research Ethics Committee of Gonabad University of Medical Sciences with the allocated code of 425. In addition, the guidelines of the National Ethics Committee of the Ministry of Health and Medical Education for the care and use of laboratory animals were observed.

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