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Metformin attenuates myocardial remodeling and neutrophil recruitment after myocardial infarction in rat

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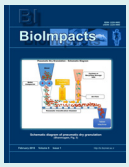

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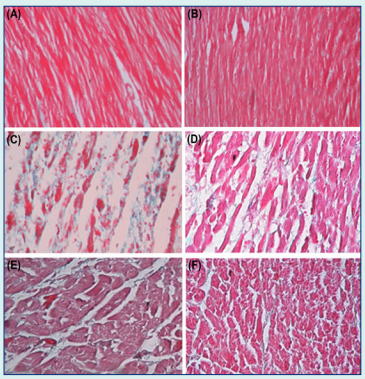
Abstract



Introduction: Acute treatment with metformin has a cardio-protective effects by suppression of inflammatory responses during myocardial infarction (MI) through activation of AMP-activated protein kinase (AMPK). Neutrophils have a pivotal role during MI-induced inflammatory responses. Some anti-inflammatory treatments have decreased cardiac injury and infarct size in MI. Here we evaluated the effects of chronic pre-treatment with metformin on myocardial remodeling and neutrophil recruitment after isoproterenol-induced MI.

Methods: Male wistar rats were randomly assigned into 6 groups (n=6) of untreated control, sham, isoproterenol (Iso), and pre-treated orally with 25, 50, and 100 mg/kg of metformin, twice daily, for 14 days. Isoproterenol was injected subcutaneously (*sc*) at 13th and 14th days for induction of acute MI. Histopathological examinations were done on the harvested hearts. Number of neutrophils in peripheral blood and their infiltration to myocardium were evaluated by Gimsa staining and myeloperoxidase (MPO) assay, respectively.

Results: Histopathological analysis showed a significant attenuation of isoproterenol-induced cardiomyocyte necrosis and fibrosis by all three doses of metformin. The heart to body weight ratio was also decreased with all doses of metformin. Pre-treatment with metformin in comparison to Iso (MI) group reduced peripheral neutrophils ($p<0.05$, $p<0.01$, and $p<0.001$ at 25, 50, and 100 mg/kg; respectively) as well as MPO activity ($p<0.05$ and $p<0.01$ at 50 and 100 mg/kg, respectively).

Conclusion: Pre-treatment with metformin decreased post-MI myocardial injuries by reducing cardiac remodeling and myocardial neutrophil activity. The results could be explained as a new mechanism for cardio-protective effect of metformin.

Introduction

Cardiac remodeling along with inflammatory responses is one of the main determinants of patients' outcome following MI.[1] Fibrosis, as a main component of cardiac remodeling, includes microscopic scarring which serves to replace lost contractile cells after necrosis with non-functional scar tissue and thereby plays an essential role in preserving myocardial structure. The extension of fibrosis demonstrates widespread and ongoing necrosis of cardiomyocytes.[2,3] Although there are multiple treatments to counteract left ventricular remodeling such as β-blockers and angiotensin-converting enzyme inhibitors, the incidence of congestive heart failure continues to increase and remains associated with a more than 10-fold elevated risk of death.[1,4] Long term ischemia that causes myocardial infarction is associated with inflammatory response, which is needed for myocardial fibrosis. It has been reported for the first time that anti-inflammatory treatment with corticosteroids, decreases the cardiac injury and infarct size in a canine model of experimental myocardial infarction.[5,6] The other study by Shiomi T et al showed that pioglitazone as a peroxisome

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proliferator-activated receptor gamma (PPAR-γ) agonist through suppression of inflammatory responses improved left ventricular remodeling and function in mice with post-MI heart failure.[7] Inflammatory responses as a host response to tissue injury result in cardiac remodeling after MI. Therefore the degree of inflammatory responses can affect the host outcome.[8] In this regard neutrophils play a fundamental role in the inflammatory responses to ischemia/reperfusion injury by releasing oxidants, proteases, toll like receptors (TLRs) activation, and releasing inflammatory products. These actions increase the tissue damage, which in turn maximize the recruitment and activation of greater numbers of neutrophils into the infarcted myocardium. Since neutrophils are activated and recruited to infarcted myocardium and most importantly have abundant tools such as oxidants and proteases to damage the myocardium, it is logical to consider that neutrophils have an important role in ischemia reperfusion injury.[9] In this regard, the activation of neutrophils is against host tissues such as the endothelium and myocardium and this normal response has no system of checks and balances to distinguish self-tissue from non-self to adjust itself.[9,10] Relative depletion of circulating neutrophils decreased the infarct size by 47%,[11] or one another study demonstrated a 50% reduction in myocardial infarct size using anti-neutrophil antiserum.[12] Chen et al reported that p-selectin immune-neutralization reduces the reactive oxygen species (ROS) generation because of a reduction in myocardial neutrophil accumulation.[13] Therefore neutrophils are closely participating in the pathogenesis of MI and play a pivotal role in the lethal injury following the reperfusion. Hence inhibiting the neutrophil-mediated inflammatory cascade during the reperfusion can be considered as a therapeutic target.[10] Metformin is a first-line orally used anti-diabetic agent for the treatment of Type 2 diabetes.[14] There are several studies that report metformin has cardio-protective effects and reduces mortality and cardiovascular end points of type 2 diabetes. The protective action of metformin is not attributed entirely to its anti-hyperglycemic actions.[15-17]

It has been reported that the activation of AMPK has anti-inflammatory effects.[18] Recently we have reported that the acute and short term administration of metformin as an AMPK activator attenuates MPO activity accompanied by a reduction in peripheral neutrophil count in isoproterenol induced myocardial injury.[19,20] The question is that whether the chronic administration of metformin, such as those seen in patients who are using the drug for a long time has any protection on isoproterenol induced myocardial remodelling. In this study we used isoproterenol-induced MI model in rats. Isoproterenol is a synthetic β-adernoreceptor agonist that causes cardiac hypertrophy, necrosis, interstitial cell fibrosis and ultimately infarct-like damage in its subcutaneous injection.[21] Anti-inflammatory effect of metformin has been reported but, the effect of chronic metformin pre-treatment on isoproterenol induced cardiac remodeling and inflammation is unclear. Our hypothesis was that metformin can protect infarcted myocardium through decreasing neutrophil recruitment and cardiac remodeling.

Materials and methods

Animals

Healthy adult male Wistar rats, weighting 260±20g, were used in this study. Rats were housed in polyethylene cages, six per cage, with food and water available *ad libitum* under standardized conditions (12-h light/dark cycle, temperature 22±1 °C and 50±10% humidity). All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (No 85-23, revised 1985) and approved by the Ethics Committee of Tabriz University of Medical Sciences.

Experimental procedures

The rats were randomly divided into 6 groups consisting of 6 rats each. Group 1 (control) received saline orally (0.5 ml, twice per day) for 14 days and were injected subcutaneously with saline (0.5 ml) at 13th day for two consecutive days. Group 2 (sham) were treated orally with metformin (100 mg/kg; twice per day) for 14 days and received subcutaneous injection of saline at 13th day for two consecutive days. Group 3 (MI; Iso) received saline orally (twice per day) for 14 days and at 13th day received subcutaneous injection of isoproterenol (100 mg/kg/day) for two consecutive days. Groups 4 to 6 received metformin orally, at doses 25, 50, 100 mg/kg/12h, for 14 days and were given isoproterenol the same as group 3. Metformin was dissolved in saline and was administered orally at a volume of 0.25-0.5 ml based on body weight.

Chemicals

Isoproterenol hydrochloride was obtained from Sigma Chemicals Company. Metformin was provided by Osvah Pharmaceutical Inc (Tehran, Iran). All other chemicals were of a commercial analytical grade.

Induction of myocardial infarction

Isoproterenol was dissolved in saline and injected *sc* to rats (100 mg/kg) for 2 consecutive days at an interval of 24 h to induce experimental MI. Animals were sacrificed 48 h after the first dose of isoproterenol.[19]

Tissue weights

At the end of experiment, the animals were anesthetized by pentobarbital and the hearts were removed and weighed. The wet heart weight to body weight ratio was calculated for assessing the degree of myocardial weight gain.

Histopathological examination

For the histopathological examination, a separate group of rats (n = 5) treated with isoproterenol, isoproterenol plus metformin (25, 50, and 100 mg/kg) and metformin alone (100 mg/kg) was used. The hearts were rapidly dissected out and fixed in 10% formalin. The heart samples were embedded in paraffin for preparing paraffin blocks.

Then the samples cut at 5 µm thickness and stained with Gomeri trichrome for assessing myocardial fibrosis and also stained with Hematoxylin and Eosin (H&E) for evaluation of necrosis. Myocardial fibrosis and necrosis were evaluated in each of the heart tissue sections using a morphometric point-counting procedure.[22] The histopathological changes were scored by two persons as 1, 2, 3, and 4 for low, moderate (focal myocyte damage or small multifocal degeneration with slight degree of inflammation), high (extensive myofibrillar degeneration and/or diffuse inflammatory process), and intensive (necrosis with diffuse inflammatory process) pathological changes, respectively.

Neutrophil counting in blood

In order to determine the number of neutrophils in blood, venous blood samples were collected from the portal vein. Fresh blood samples were smeared on the clean lams and the percent of neutrophils were counted at 100× zooming in optical microscope after Gimsa staining.

Myeloperoxidase assay

To quantify myocardial neutrophil infiltration the cardiac activity of MPO, an abundant enzyme of neutrophils, was assessed as previously described by Mullane et al.[23] The tissue samples were homogenized in 50 mM potassium phosphate buffer (PH 6) containing 0.5% hexadecyl-trimethyl ammonium bromide (HTAB) to solubilize the enzyme. The samples were then freeze-thawed three times and sonicated for 20 s. After that the samples were centrifuged at 11,000×g for 30 min at 4 °C, and the resulting supernatant was assayed spectrophotometrically for MPO determination. In brief, 0.1 ml of the supernatant was then allowed to react with a 2.9 ml solution of 50 mM potassium phosphate buffer (pH 6) containing 0.167 mg/ml of O-dianisidine hydrochloride and 0.0005% H_2O_2. Five minutes later, 0.1 ml of 1.2 M hydrochloric acid was used to stop the reaction. Then the change rate in absorbance at 460 nm was measured by a spectrophotometer (Cecil 9000, UK). The activity of myeloperoxidase (MPO) are presented as miliunits of MPO in gram tissue.

Statistical analysis

The results are expressed as mean±SEM and were analyzed using one-way-ANOVA to make comparisons between the groups. If the ANOVA analysis indicated significant differences, a Student–Newman–Keuls *post-hoc test* was used for pair wise comparison. The level of significance was set at $p<0.05$.

Results

Effect of chronic pre-treatment with metformin on histophathology of infarcted myocardium

The regularly arranged myocardial fibers with clear striations were observed in the control group (Figs. 1-3). Moreover, there was no evidence of necrosis or fibrosis in the control group. The histological sections of the hearts obtained from isoproterenol treated rats showed

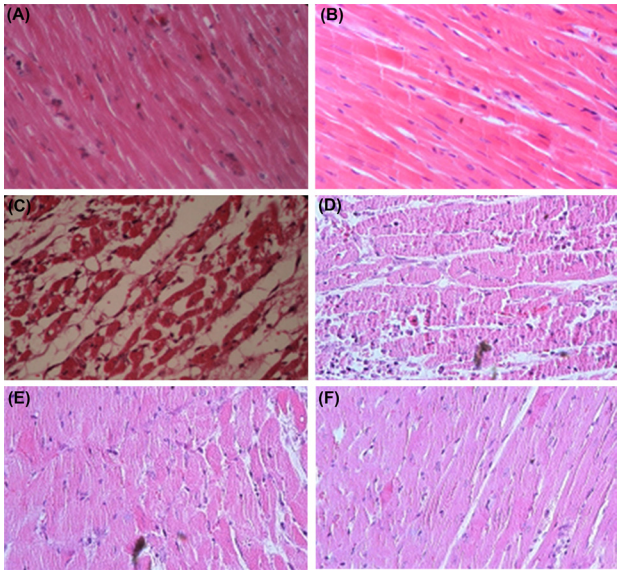

Fig. 1. Photomicrographs of sections of rat cardiac apexes for evaluating necrosis. Heart tissue of a rat treated with isoproterenol (Iso) shows intensive cardiomyocyte necrosis and increased edematous intramuscular space. Chronic pre-treatment with metformin (Met) demonstrates a marked improvement. Iso: isoproterenol; Met: metformin. H&E (40× magnification). (A) Control; (B) Sham (Met 100 mg/kg); (C) isoproterenol; (D) Iso+Met (25 mg/kg/day); (E) Iso+Met (50 mg/kg/day); (F) Iso+Met (100 mg/kg/day).

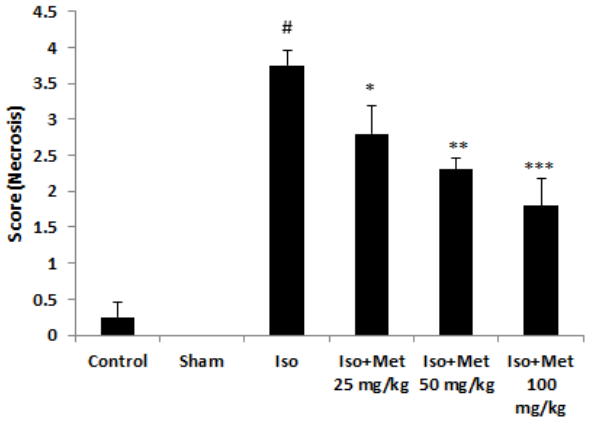

Fig. 2. Grading of histopathological changes in the rat's cardiac apex tissues that was stained with H&E. Grades 1, 2, 3, and 4 show low, moderate, high and intensive pathological changes, respectively. Iso: isoproterenol; Met: metformin. Values are mean±SEM (n=5). #$p<0.001$ as compared with control. *$p<0.05$, ** $p<0.01$, *** $p<0.001$ as compared with isoproterenol treated group using one way ANOVA with Student-Newman-Keuls *post hoc* test.

widespread subendocardial necrosis, hypertrophy, and abundant fibroblastic hyperplasia (Figs. 1 and 3). Oral administration of metformin at doses of 25, 50, and 100 mg/kg was started 12 days before induction of infarction and continued for two days at an interval of 12 h after first isoproterenol injection, significantly prevented inflammatory responses and myocardial fibrosis and necrosis. It was observed that the chronic pre-treatment with metformin at doses of 25, 50, and 100 mg/kg reduced

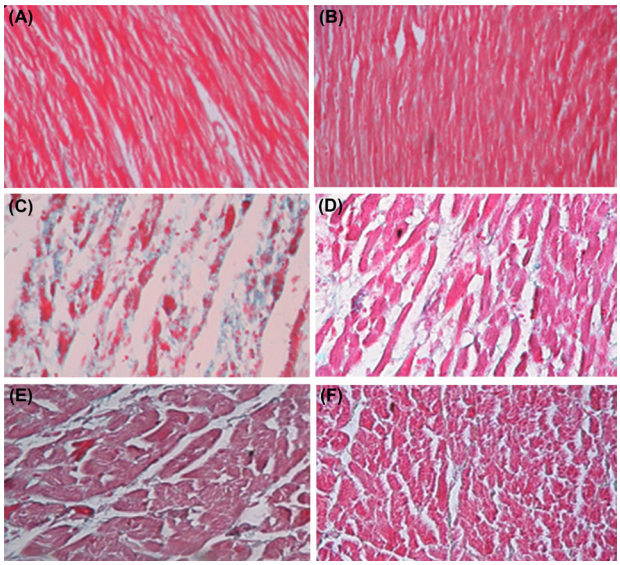

Fig. 3. Photomicrographs of sections of rat cardiac apexes for showing fibrosis. Heart tissue of a rat treated with isoproterenol (Iso) shows intensive cardiomyocyte fibrosis. Chronic pre-treatment with metformin (Met) demonstrates a marked improvement. Iso: isoproterenol; Met: metformin. Gomeri's one-step trichrom method (40× magnification). (A) Control; (B) Sham (Met 100 mg/kg); (C) isoproterenol; (D) Iso+Met (25 mg/kg/day); (E) Iso+Met (50 mg/kg/day); (F) Iso+Met (100 mg/kg/day).

the isoproterenol-induced necrosis (Fig. 2) from 3.75±0.2 in dose-dependent manner by 25% (2.8±0.4) (p<0.05), 39% (2.3±0.16) (p<0.01), 52% (1.8±0.38) (p<0.001), and also reduced isoproterenol-induced fibrosis (Fig. 4) from 3.5±0.2 by 26% (2.5± 0.2), 27% (2.5±0.2) (p<0.01), and 32% (2.3±0.2) (p<0.001), respectively. Metformin alone (sham) had no effect on tissue stricter and histology in normal healthy animals as shown in Figs. 1 and 3.

Effect of chronic pre-treatment with metformin on the heart weight gain

The heart weight to body weight ratio was determined to assess the extent of heart weight gain developed by injection of isoproterenol (Fig. 5). The ratio was significantly higher in the isoproterenol treated rats (4.54±0.32) compared with the control group (2.65±0.07; p<0.001). Chronic pre-treatment with 25, 50 and 100 mg/kg of metformin for 14 days produced a substantial (p<0.05 and p<001) reduction in the heart to body weight ratio in comparison to the isoproterenol alone treated rats (Fig. 5). No significant difference was observed in the heart weight of healthy rats pre-treated with metformin alone (100 mg/kg; sham) when compared to the normal control rats.

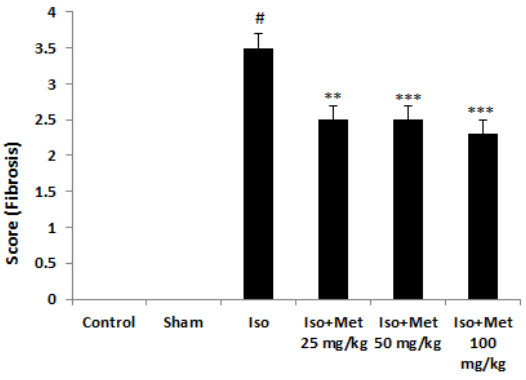

Fig. 4. Grading of histopathological changes in the rat's cardiac apex tissues which was stained with Gomeritrichrome. Grades 1, 2, 3, and 4 show low, moderate, high and intensive pathological changes, respectively. Iso: isoproterenol; Met: metformin. Values are mean±SEM (n=5). #p<0.001 as compared with control. **p<0.01, ***p<0.001 as compared with isoproterenol treated group using one way ANOVA with Student-Newman-Keuls *post hoc* test.

Effect of Chronic pre-treatment with metformin on the activity of myocardial MPO and neutrophil count in blood following MI

Neutrophil infiltration into myocardial tissue significantly

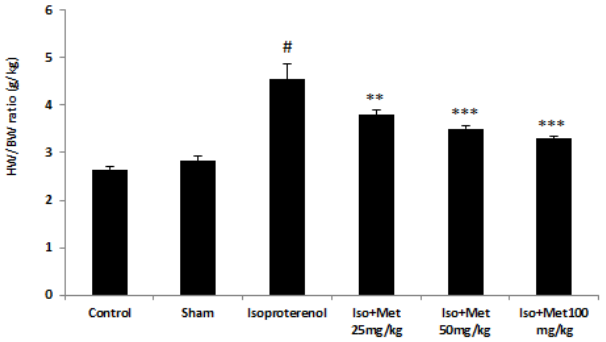

Fig. 5. Heart weight (HW) to body weight (BW) ratio in the control group and in the rats treated with isoproterenol alone (rats with myocardial infarction), metformin alone (100 mg/kg, sham), and with isoproterenol plus metformin. Iso: isoproterenol; Met: Metformin. Values are mean±SEM (n=6). #p<0.001 from respective control value; *p<0.05 and ***p<0.001 as compared with isoproterenol treated group using one way ANOVA with Student-Newman-Keuls *post hoc* test.

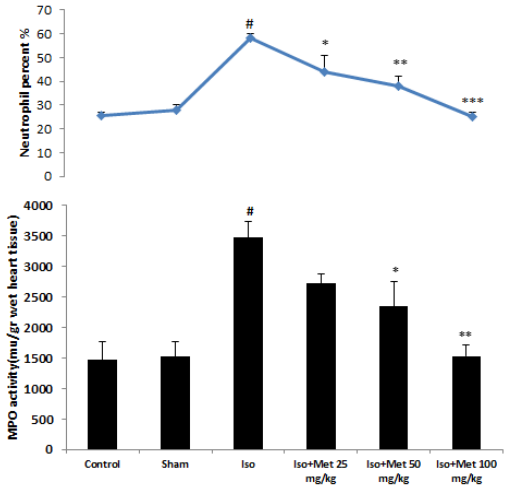

Fig. 6. The effect of chronic pre-treatment with metformin (Met) at the doses of 25, 50, and 100 mg/kg/12 h (for 14 days) on MPO activity in infracted myocardium (bar graphs) and on neutrophil count in blood (line graph). Values are mean±SEM (n=6). #p<0.01, from respective control value; *p<0.05, **p<0.01, ***p<0.001 as compared with isoproterenol (Iso) injected group using one way ANOVA with Student-Newman-Keuls *post hoc* test.

(p<0.01 vs. control) increased in Isoproterenol alone (MI) group and this was accompanied by an increase in MPO activity in the myocardium (Fig. 6). Chronic pre-treatment with metformin at the doses of 25, 50, and 100 mg/kg/12h for 14 days reduced the myeloperoxidase activity in the left ventricle from 3469±275 mU/g wet tissue in isoproterenol group (MI) to 2719±159, 2348±407 (p<0.05), and 1534±189 (p<0.01) mU/g, respectively (Fig. 6). Metformin-induced reduction in activity of MPO was associated with a similar decline in the peripheral neutrophil percentage, which was significantly (p<0.001) elevated from 25.5±6% in control to 58±1% by isoproterenol (MI).

Research Highlights

What is current knowledge?

√ Anti-inflammatory effect ofmetformin has been reported but the effect of chronic metformin pre-treatmenton isoproterenol induced cardiac remodeling and inflammation is unclear.

What is new here?

√ Cardio-protective effects of metformin is mediated at least in part byreduced neutrophil infiltration to myocardial tissue as well as reduction incardiac remodelling.

Discussion

The present study demonstrates that pre-treatment with metformin alleviates cardiac necrosis and fibrosis, and neutrophil infiltration to myocardial tissue after isoproterenol induced MI. Recently several experimental and clinical studies have reported that metformin possesses cardio-protective effects beyond its glucose-lowering properties. However, metformin's possible mechanisms of action beyond glycemic control remain poorly understood. United Kingdom Prospective Diabetes Study (UKPDS) Group has shown that treatment of diabetic patient with metformin reduces the risk for any diabetes-related endpoint by 32% and 36% for all-cause mortality and also is associated with a 39% lower risk of MI.[16] A study by Zhao et al showed that chronic pre-treatment with metformin in diabetic patients may reduce no-reflow and consequently improve outcome in patients suffering from an acute MI.[17]

One of the major strategies to overcome the injuries after MI is minimizing myocardial necrosis and optimizing cardiac repair following MI. A large number of studies suggested a role for a variety of inflammatory responses in MI and several experimental studies have shown a significant reduction in infarct size with the use of specific anti-inflammatory strategies.[3,5] Different studies suggest that MI-induced myocardial inflammation and injury may be neutrophil dependent.[9] A study showed that neutrophil depletion in dogs undergoing ischemia-reperfusion led to a marked decrease in infarct size.[24] The pleotropic effects of metformin may be mediated by its activation of AMPK. A study by Calvert et al demonstrated that acute administration of metformin protects cardiac tissue through AMPK dependent pathway.[25] Although metformin through activation of AMPK decreases protein synthesis in cardiac myocyte and therefore probably post-MI hypertrophy,[26] it has been also reported that AMPK is present in neutrophils and may affect their pro inflammatory activities.[27] However, it is unknown whether metformin protect the infarcted myocardium by inhibiting neutrophil recruitment and activity. In the present study pre-treatment with metformin decreased cardiac necrosis and fibrosis following isoproterenol-induced MI in rats and these beneficial effects were associated with a significant reduction in the number and activity of neutrophils in heart tissue. To the best of our knowledge, this is the first study showing that chronic pre-treatment with metformin might also have beneficial effects against myocardial neutrophil activity and cardiac remodeling. There are some reports in which metformin treatment may be associated with beneficial clinical outcomes in patients with acute coronary syndrome[28] and in patients undergoing coronary angioplasty.[29] In summary, we believe that cardio-protective effects of metformin is mediated at least in part by reduced neutrophil infiltration to myocardial tissue as well as reduction in cardiac remodelling.

Acknowledgments

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Ethical issues

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Tabriz University of Medical Sciences, Tabriz-Iran (National Institutes of Health publication No 85-23, revised 1985).

Competing interests

The authors declare no conflict of interests.

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