

## Protective Effect of Metformin on Cardiomyocytes Ischemia-Reperfusion (IR) Induced Apoptosis in Rats

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**Abstract:** The heart failures following infarctions is one of the most important causes of death throughout the world. This study was undertaken to investigate the protective effects of metformin on apoptotic cell death of cardiomyocytes during experimental cardiac Ischemia-Reperfusion (IR) in rats. The 25 male Wistar rats were randomly assigned into 5 groups of 5 animals each including; Sham/IR, IR, low dose metformin+IR, average dose metformin+IR and high dose metformin+IR. Heart muscle ischemia was induced clamping the left descending coronary artery. After 30 min of ischemia, the clamps were taken off and the animals underwent 2 h reperfusion. Metformin (5, 10 and 20 µg/kg/min) was infused 15 min prior to reperfusion through jugular vein in treatment groups. At the end of experiment, the rats were euthanized and histological sections from left ventricles were prepared through Tunnel Staining method. Apoptotic cells were counted under light microscope. The data obtained were statistically analyzed using ANOVA. Differences were considered statistically significant at  $p < 0.05$ . In group 2, ischemia-reperfusion caused occurrence of apoptotic cell death in cardiomyocytes. There was a significant increase in the incidence rate of apoptosis of cardiomyocytes in comparison with group 1 ( $p < 0.001$ ). In groups 3-5 metformin (5, 10 and 20 µg/kg/min) caused significant decrease in the number of apoptotic cells in comparison with group 2 ( $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively). This study therefore, suggests that metformin may be a useful agent for the prevention of Ischemia-Reperfusion (IR) induced apoptotic cell death of cardiomyocytes in a dose dependent manner in the rats.

**Key words:** Ischemia-reperfusion, cardiomyocytes, metformin, apoptosis, rat, Iran

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### INTRODUCTION

Ischemia-reperfusion or IR for short is a complex clinical syndrome that under certain conditions can affect different organs. It includes a phase of decrease or stop in the blood perfusion of the organ and after a while causes the reperfusion to the organ (Gottlieb and Engler, 1999). If the cells are damaged in revocable ways, the revival of blood reperfusion can lead to repairing the damaged cells. Meanwhile under certain circumstances, ischemia-reperfusion to the damaged cells that are healthy in all other aspects paradoxically causes more intense damage in them. Consequently in addition to the cells damaged irrevocably during ischemic period, some other cells are destroyed as well. This state called as ischemia-reperfusion damage is an important clinical process that has a major role in tissue destruction. However, it can be controlled by taking some corrective treating action. Although, the exact mechanisms for this

type of injury have not been identified, one of the major reasons behind IR can be summarized in the following way; blood reperfusion intensifies the local absorption of the inflammatory cells. These cells release a considerable amount of active oxygen radicals causing cell membrane damage and mitochondria permeability. Higher mitochondria permeability and the formation of tiny openings on its membrane lower the membrane potential and decrease the production of ATP resulting in inflated mitochondria. Higher outer mitochondria permeability causes the release of cell damage stimulators (Gottlieb and Engler, 1999). 5-AMP-activated protein Kinase (AMPK) is one of the key energy metabolism regulators in heart and other tissues. During metabolic stress like hypoxia AMPK is activated. It not only causes the activation of energy producing paths but it also controls some of the energy consuming paths (Hardie and Carling, 1997). Today, AMPK is considered as the energy sensor inside the cell, especially in heart cells that have high and

constant need for ATP. The stimulation AMPK paths in fact, increase both glucose and fatty acid in case of higher demand for them. The role of AMPK as an energy sensor, especially in ischemic and hypoxia is very important. Although, it has been demonstrated that AMPK has a significant role in the regulation of metabolism inside fatty acid and glucose cells (Dyck and Lopaschuk, 2006), some new evidence indicate that AMPK besides regulating the energy has a significant role in balancing severe inflammatory reactions. For example in endothelial cells, AMPK stimulation is accompanied by the control on cytotoxicity TNF- $\alpha$  (Su *et al.*, 2007). Inflammatory cells have important roles in pathophysiologic responses to ischemic-reperfusion related damage like that of heart muscle infraction. Macrophages and neutrophils are the major leucocytes in developing and intensifying infraction after reperfusion. Immediately after ischemic, monocytes and the other leucocytes penetrate the ischemic region. The activated macrophages release cytokines that intensify the accumulation of neutrophils and tissue injury. Moreover, the penetration of neutrophils to ischemic tissue by releasing proteolysis and free radicals intensifies tissue necrosis and expands the infraction region (Frangogiannis *et al.*, 2002). AMPK is present in neutrophils and macrophages. It has been proved that the stimulation of AMPK reduces the activity of the mentioned cells. It has also been demonstrated that it increases the production of ATP in heart ischemic stresses and controls apoptosis (Dyck and Lopaschuk, 2006). Metformin is a highly effective and frequently used drug in curing diabetic and metabolic illnesses. Some of its curing and effective uses are due to the activation of AMPK.

It is especially noted today in research as an AMPK path stimulator (Dyck and Lopaschuk, 2006). Considering the fact that metformin is effective in AMPK route, the present research was conducted with the aim of studying the cytoprotective effects of metformin on cardiomyocytes ischemia-reperfusion induced apoptosis.

## MATERIALS AND METHODS

The present study is an experimental laboratory based research conducted on rats in 2011 in research center of Islamic Azad University, Tabriz Branch. All ethical considerations and work protocols on lab animals was approved by lab animals Supervisory Committee of Research Center for Islamic Azad University. For the present study, 25 male 10 weeks old Wistar rats weighing  $230 \pm 20$  g were obtained from Iran Pastor Institute Lab Animal's Center. They were randomly

allocated into 5 groups of 5 animals each, namely; Sham/IR, IR, low dose metformin ( $5 \mu\text{g kg}^{-1}$ ) +IR, average dose metformin ( $10 \mu\text{g kg}^{-1}$ ) +IR and high dose metformin ( $20 \mu\text{g kg}^{-1}$ ) +IR. The conditions for all groups were equally the same having 12 of light/dark hours at a temperature of  $20 \pm 2^\circ\text{C}$  in special cages on beds of straw. Food and water were freely provided. After a week of adjustment to the new conditions, the study was conducted on rats. Metformin was purchased from Sigma Business group. The drug dosage was calculated based on the findings of Calvert *et al.* (2008), Solskov *et al.* (2008) and Gundewar *et al.* (2009). The different dosages of the drugs in DMSO solvents of 2.5% were solved in Saline. About 2 h before the operation, food was withheld from all groups. For the purpose of making the animals unconscious,  $50 \text{ mg kg}^{-1}$  of Ketamin (Ketamin 10%, Alfasan, Woerden and Holland) and  $5 \text{ mg kg}^{-1}$  of Xylazin (Xylazin 2%, Alfasan and Woerden, Holland) were used in peritoneal way. Jugular vein was canalized for drug and unconsciousness agent infusion and carotid artery for recording Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP) and Heart Beat (HR). The animals were operated following Garjani *et al.* (1995). After 10 min of stabilization to create ischemia, the left lower coronary was blocked with a clamp. After 30 min, the clamps were removed and ischemic reperfusion was maintained for 2 h. Total 15 min prior to reperfusion, metformin ( $5, 10$  and  $20 \mu\text{g kg}^{-1}$ ) was infused into the treatment group rats through jugular vein.

In Sham/IR and IR groups instead of metformin, normal saline was injected in the same manner. The rate of infusion was kept constant for all groups. Lead-I ECG standard during experiment and arrhythmia during ischemia and the 1st 30 min of reperfusion was analyzed and counted based on Lambth protocol (Garjani *et al.*, 1995). In all groups, physical signals such as dyspnea and tachypnea were studied in terms of their occurrence and intensity. At the end of the experiment to do microscopic analysis, the rats were euthanized and histological sections from left ventricles were prepared through Tunnel Staining method. After being stabilized in 10% buffer formalin, they were sent to pathological laboratory of Islamic Azad University Tabriz Branch. Consequent, sections from left ventricles of  $5 \mu$  and special tunnel coloring were prepared. The cells that were positive in terms of the existence of apoptosis were counted and analyzed with light microscope. For each count in each section of the apoptosis cells, 5 microscopic fields were randomly selected and were studied with a magnified view of  $\times 40$ .

Apoptosis was evaluated via the terminal deoxynucleotidyl transferase-mediated dUTP Nick-End

Labeling (TUNEL) method with the use of *in situ* cell death detection kit, POD (1684817, Roche, Germany) according to manufacturer's instructions with some modifications (Garjani *et al.*, 1995). Briefly, the tissue sections were dewaxed and rehydrated by heating at 60°C followed by washing in xylene and rehydration through a graded series of ethanol and double distilled water. Then, the sections were incubated for 30 min at 21-37°C with Proteinase K working solution (20 µg mL<sup>-1</sup> in 10 mM Tris-Cl, pH 7.6). The sections were rinsed with PBS and incubated with the TUNEL reaction mixture for 1 h at 37°C in a humidified chamber. As a positive control, sections were treated with DNase I (1 mg mL<sup>-1</sup>; sigma) for 10 min to introduce nicks in the genomic DNA. After converter Peroxidase (POD) was added, the sections were incubated for 30 min at 37°C in a humidified chamber. Then, the 3, 3-diaminobenzidine substrate was added for the visualization of nuclei with DNA nick end labeling. The sections were counter-stained with toluidine blue to show normal nuclei. The percentage of myocytes with DNA nick end labeling was analyzed by counting the cells exhibiting brown nuclei a ×40 magnification in 5 randomly chosen fields (1 mm<sup>2</sup>) in triplicate plates.

The number of TUNEL-positive cardiomyocytes was counted by double-blinded observation. The quantitative data for the present study were shown in the form of (Mean±SEM) and the significant differences between the groups were analyzed using ANOVA and Tukey. The differences were significant at p<0.05.

**RESULTS AND DISCUSSION**

Results of the effect of graded doses of metformin on the incidence rate of apoptotic cell death of cardiomyocytes induced by ischemia-reperfusion are shown in Fig. 1. In IR group, ischemia-reperfusion caused severe apoptotic changes of cardiomyocytes as apoptotic cells were observed in light to dark brown color (Fig. 2).

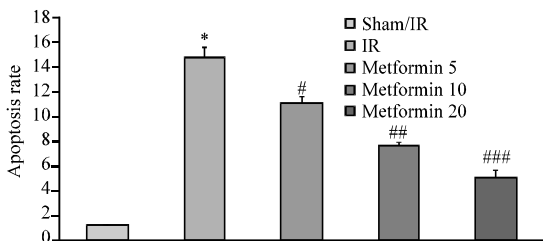


Fig. 1: Comparative evaluation of apoptosis occurrence of cardiomyocyte among the controls and treatment groups; \*significantly different from the Sham/IR group \*p<0.001; #Significantly different from the IR group #p<0.05, ##p<0.01, ###p<0.001

The incidence rate of apoptotic cell death in this group was significantly higher than Sham/IR group (p<0.001). In group 3, metformin (5 mg kg<sup>-1</sup> IV infusion/min) significantly (p<0.05) reduced apoptosis of cardiomyocytes induced by ischemia-reperfusion compared to IR group. In group 4, metformin (10 mL kg<sup>-1</sup> IV infusion/min) caused significant (p<0.01) reduction of ischemia-reperfusion induced apoptosis of cardiomyocytes in comparison with IR group. In group 5, metformin (20 mL kg<sup>-1</sup>, vein infusion) caused a significant (p<0.001) reduction of ischemia-reperfusion induced apoptotic cell death of cardiomyocytes comparing IR group. However, even this dosage of the metformin could not normalize the changes in apoptosis and there still was a significant difference between this group and IR/Sham (p<0.05) (Fig. 1-5).

In this study, revealed that metformin caused decrease in apoptosis in heart muscle cells due to

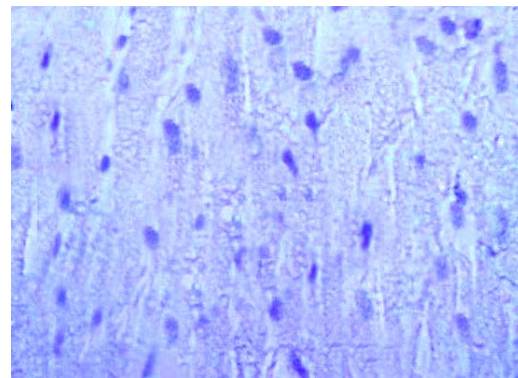


Fig. 2: Microscopic view of heart tissue of rat from healthy control group in which the normal structure of the heart is visible (TUNEL method, ×40)

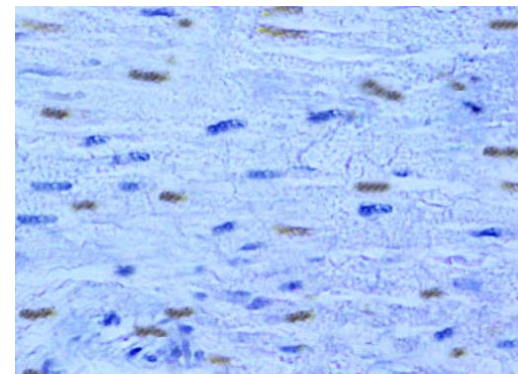


Fig. 3: Microscopic view of heart tissue in rats from diseased control group. Positive tunnel cells are frequently visible in brown (TUNEL method, ×40)

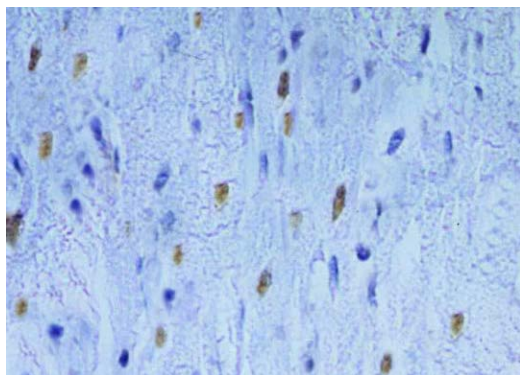


Fig. 4: Microscopic view of heart tissue in rats from 5 mg/kg/min dosage metformin treatment group. Positive tunnel cells are frequently visible in brown (TUNEL method, ×40)

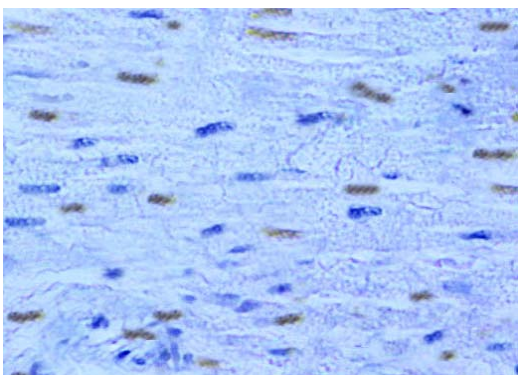


Fig. 5: Microscopic view of heart tissue from a rat of IR group. Positive tunnel cells are frequently visible in brown (TUNEL method, ×40)

ischemic-reperfusion so that was related to administrated dose. Other researchers results also showed that high dose of metformin have more cytoprotective effect than mild and low doses (Solskov *et al.*, 2008). Metformin is effective in treatment of diabetic patients and metabolic disorders (Jermendy, 2010). Part of the therapeutic effects of metformin is related to AMPK activation so that now a days this drug is used in medical research as a stimulating of AMPK pathway (Calvert *et al.*, 2008). There are a few studies on effect of metformin on injuries subsequent ischemic reperfusion. Legtenberg *et al.* (2002) showed that short-term infusion of metformin (12 min) during a mild ischemia improves heart performance after ischemia on rats. Bhamra *et al.* (2008) and Paiva *et al.* (2009) showed that infusion of metformin ( $50 \mu\text{mol L}^{-1}$ ) for 15 min on normal and diabetic hearts reduced infarcted

area size. They planned the inhibition of MPTP (Mitochondrial Permeability Transition Pore opening) and excitation of adenosine receptors as action mechanisms. In all done studies about protective effect of metformin as single dose or low dose or short-term usage on injuries subsequent ischemic-reperfusion and heart failures suggested that activation of AMPK pathway is as metformin action mechanism (Solskov *et al.*, 2008; Calvert *et al.*, 2008; Gundewar *et al.*, 2009). Sasaki *et al.* (2009) demonstrated that metformin by reduction of oxidative stress prevented of apoptosis in heart muscle cells of dog's subsequent ischemic-reperfusion. They also declared that metformin by activation of AMPK plays an important protective role on heart subsequent ischemic-reperfusion. Russell *et al.* (2004) revealed that metformin by activation of AMPK reduce the occurrence of apoptosis in heart muscle cells during ischemic reperfusion and finally heart failure is prevented. Their findings are consistent with current research results. An *et al.* (2006) concluded that metformin through inhibition of caspase 3 enzyme activity prohibits of palmitic acid effects on apoptosis induction on heart muscle cells. Calvert *et al.* (2008) expressed that metformin through ENOS pathway (Endothelial Nitric Oxide Synthase pathway) applies its protective role on heart ischemic-reperfusion. Solskov *et al.* (2008) showed metformin cytoprotective effect by oral administration of metformin ( $250 \text{ mg kg}^{-1}$ ) as single dose, 24 h before heart ischemic-reperfusion.

## CONCLUSION

The study shows that metformin protects of heart against ischemic-reperfusion through inhibition of apoptosis occurrence in muscular fibers. Considering the above mentioned series, it can be declared that metformin can use as a preventive or harmless therapeutic agent against damages of cardiac ischemic-reperfusion after controlled clinical trials. Thus to understanding of accurate sites and action mechanism of metformin requires many more expanded studies.

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## REFERENCES

- An, D., G. Kewalramani, J.K. Chan, D. Qi and S. Ghosh *et al.*, 2006. Metformin influence cardiomyocyte cell death by pathway that are dependent and independent of caspase-3. *Diabetologia*, 49: 2174-2184.
- Bhamra, G.S., D.J. Hausenloy, S.M. Davidson, R.D. Carr and M. Paiva *et al.*, 2008. Metformin protects the ischemic heart by the Akt-mediated inhibition of mitochondrial permeability transition pore opening. *Basic Res. Cardiol.*, 103: 274-284.
- Calvert, J.W., S. Gundewar, S. Jha, J.J.M. Greer, W.H. Bestermann, R. Tian and D.J. Lefer, 2008. Acute metformin therapy confers cardioprotection against myocardial infarction via AMPK-eNOS-mediated signaling. *Diabetes*, 57: 696-705.
- Dyck, J.R. and G.D. Lopaschuk, 2006. AMPK alterations in cardiac physiology and pathology: Enemy or ally? *J. Physiol.*, 574: 95-112.
- Frangogiannis, N.G., C.W. Smith and M.L. Entman, 2002. The inflammatory response in myocardial infarction. *Cardiovas Res.*, 53: 31-47.
- Garjani, A., C.L. Wainwright, I.J. Zeitlin, C. Wilson and S.J. Slee, 1995. Effects of endothelin-1 and the ETA-receptor antagonist, BQ123, on ischemic arrhythmias in anesthetized rats. *J. Cardiovasc Pharmacol.*, 25: 634-642.
- Gottlieb R.A. and R.L. Engler, 1999. Apoptosis in myocardial ischemia-reperfusion. *Ann. N. Y. Acad Sci.*, 874: 412-426.
- Gundewar, S., J.W. Calvert, S. Jha, I. Toedt-Pingel and S.Y. Ji *et al.*, 2009. Activation of AMP-activated protein kinase by metformin improves left ventricular function and survival in heart failure. *Circ. Res.*, 104: 403-411.
- Hardie, D.G. and D. Carling, 1997. The AMP-activated protein kinase-fuel gauge of the mammalian cell? *Eur. J. Biochem.*, 246: 259-273.
- Jermendy, G., 2010. New clinical data with metformin therapy in patients with diabetes mellitus. *Orv. Hetil.*, 151: 2025-2030.
- Legtenberg, R.J., R.J. Houston, B. Oeseburg and P. Smits, 2002. Metformin improves cardiac functional recovery after ischemia in rats. *Horm Metab. Res.*, 34: 182-185.
- Paiva, M., N.P. Riksen, S.M. Davidson, D.J. Hausenloy and P. Monteiro *et al.*, 2009. Metformin prevents myocardial reperfusion injury by activating the adenosine receptor. *J. Cardiovasc. Pharmacol.*, 53: 373-378.
- Russell, R.R., J. Li, D.L. Coven, M. Pypaert and C. Zechner *et al.*, 2004. AMP activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis and injury. *J. Clin. Invest.*, 114: 495-503.
- Sasaki, H., H. Asanuma, M. Fujita, H. Takahama and M. Wakeno *et al.*, 2009. Metformin prevents progression of heart failure in dogs: Role of AMP-activated protein kinase. *Circulation*, 119: 2568-2577.
- Solskov, L., B. Lofgren, S.B. Kristiansen, N. Jessen and R. Pold, 2008. Metformin induces cardioprotection against ischaemia/reperfusion injury in the rat heart 24 hours after administration. *Basic Clin. Pharmacol. Toxicol.*, 103: 82-87.
- Su, R.Y., Y. Chao, T.Y. Chen, D.Y. Huang and W.W. Lin, 2007. 5-Aminoimidazole-4-carboxamide riboside sensitizes TRAIL- and TNF $\alpha$ -induced cytotoxicity in colon cancer cells through AMP-activated protein kinase signaling. *Mol. Cancer Ther.*, 6: 1562-1571.