New Perceptions in the Cardioprotective Effect of Metformin Against Isoproterenol Induced Cardiotoxicity in Male Rats

1Yahya Al-Qaisy, 2I. S. Arif, and 3M. Ibraheem
1&2University of Uruk College of Pharmacy. Baghdad. Iraq
3Mustansyria University. College of Medicine. Baghdad. Iraq
yahya.gh.alqaisy@gmail.com

Abstract Background and objective: Recent clinical trials have shown that metformin improves clinical cardiovascular outcome in type-2 diabetic patients independently of its insulin-sensitizing effect. This study was sought to evaluate the potential cardioprotective effects of metformin on isoproterenol-induced cardiac stress in diabetic and non-diabetic rats. Materials and Methods: Diabetes was induced by using streptozocin (60 mg/kg, i.p.) while non-diabetic rats received saline. Rats in both experimental groups were then randomized to receive different doses of metformin (75, 150, 300 mg/kg i.p.) for 6 weeks. Cardiac stress was induced by isoproterenol (150 mg/kg i.p.) for two successive days. Specific biomarkers of tissue injury, namely brain natriuretic peptide (BNP), cardiac troponin-T (cTn-T), matrix metalloproteinase (MMP), tissue necrotic factor-α (TNF-α), were assessed. Data were analysed using one-way ANOVA followed by Newman Keuls post hoc test Results: The results showed that metformin significantly limited isoproterenol-induced myocardial injury in both diabetic and non-diabetic rats. Metformin significantly decreased the elevated serum levels of brain natriuretic peptide (BNP), matrix metalloproteinase (MMP), cardiac troponin T (cTn-T) which was induced by isoproterenol. It also limited expression of tissue necrotic factor-α (TNF-α) following the cardiac injury in diabetic and non-diabetic rats.

Keywords: Metformin, Type 2 diabetes mellitus, cardio-protection, Isoproterenol-induced cardiac stress, Brain natriuretic peptide, Matrix metalloproteinase, cardiac troponin type T, Tumour necrotic factor-α.

1. Introduction

Metformin is one of the most prescribed anti-hyperglycaemic drugs used to control blood glucose level in type-2 diabetic patients. In addition to that, it has been reported that the therapeutic applications of metformin could not be limited to control diabetes but could also have some beneficial cardioprotection effects [1,2]. Clinically, it has been reported that metformin has a direct cardioprotection effects and reduces the mortality rate in diabetic patients independently of its’ glycaemic control effect [3,4]. Several mechanisms of action by which metformin might induce its cardio-protection have been suggested including activation of 5’-adenosine monophosphate-activated protein kinase (AMPK) [5,6]. Salt’s laboratory demonstrated that activation of AMPK with 5’-aminoimidazole-4-carboxamide ribonucleoside enhanced the phosphorylation of endothelial nitric oxide synthase (eNOS) at its activation site (Ser1177) in a dose dependent manner in human aortic endothelial cells [7]. It has also been demonstrated that metformin improved cellular antioxidant gene expression to mitigate cardiac remodelling following myocardial infarction [8]. Like wise, it has been reported that metformin attenuated the expression of collagen following myocardial infarction [9]. Moreover, there is an increasing body of evidence suggests that metformin therapy has anti-atherogenic activity via suppressing the expression of endothelial adhesion molecules in in vitro and in vivo studies [10]. Clinically, metformin is shown to enhance endothelium-dependent microvascular blood flow and improve symptoms of myocardial infarction (MI) presented by 38% decrease in maximal ST-segment depression and 30% decrease in the occurrence of chest pain [11]. Additionally, it has been shown that metformin treatment improved deteriorated lipid profile in type-2 diabetic patients [12]. Interestingly, metformin treatment effectively improved cardiac contractility and remodeling in ischemia-induced heart failure model in rat [9].

The field of cardio-protection has failed so far to introduce a drug which can prevent cardiac injury that occur during acute myocardial infarction, despite the extensive efforts over the last two decades. There are many reasons for this failure which have been discussed in more details in recent review papers [13,14]. One of the most important reasons probably is that the experimental model, which is employed to characterize the cardio-protection of the drug under investigation, does not reflect the clinical situation of patients with ischemia heart disease. Majority of those patients often have other comorbitides such as diabetes and hypocholesteremia, heart failure and age [13]. Therefore, this complexity needs to be taken into consideration when designing experimental cardioprotection studies. This study investigated the potential cardioprotective effects of metformin against cardiac injury in diabetic and non-diabetic rats. This study hypothesized that metformin can limit myocardial injury in the presence and absence of diabetes as a comorbidity using a rat model of isoproterenol-induced cardiac injury in vivo.
2. Materials and Methods

Animals Male Wister rats weighted 120–180 g was used in this study. Rats were housed with free access to water and small rodent’s food at a constant temperature (20°C - 25°C) and humidity for 10 days before any experimentation in a 12hr light/dark cycle in the Animals Facility at Al-Mustansiriya College of Pharmacy. All experimental protocols were carried out at the laboratories of post-graduate students/college of pharmacy/ Almustansiriya university (May 2016 – Dec. 2016) to All handling and procedures were approved by the Animals Welfare Committee in the College of Pharmacy, Al-Mustansiriya University.

Induction of diabetes Streptozocin (STZ) was freshly prepared and administered (60 mg/kg, i.p.) as a single dose. Fasting blood sugar (FBS) level was monitored (using ONETOUCH glucometer) for 10 days and only the rats with FBS >250 g/dl were considered as diabetic and included in the study.

Induction of cardiac stress: Isoproterenol (ISO) solution was prepared freshly, and rats received ISO (150 mg/kg, i.p.) for two days to induce cardiac stress.

Experimental design Male Waster rats were randomized into two main groups: diabetic and non-diabetic group. Rats in the diabetic group received STZ (60 mg/kg, i.p.) as a single dose to induce diabetes while non-diabetic rats received saline. The whole experimental protocol is shown in (Figure 1).

Materials and chemicals

Streptozocin and isoproterenol are purchased from Sigma-Aldrich, Gillingham, UK. Metformin was sourced from Pioneer Pharmaceutical Company, Sulaymaniya, Iraq. ELISA kits for (BNP, MMP-1, cTn-T) were purchased from Shanghai YeHua Biological Technology, Shanghai, China. Immunohistochemistry kit for TNF-α (MaxTag™ Histo IHC Kit) was purchased from (Rockland-inc, USA).

Samples collection Blood sample was collected from the left ventricle by heart puncture technique after anesthetized the rats with diethyl ether. Blood sample was centrifuged with 3000rpm for 6 mins and the serum was recovered and stored at -20 °C until required.

Tissue sampling and processing the heart was harvested immediately after collecting the blood sample and washed with saline to remove any blood residue. The heart was then fixed with 10% formalin (pH 7.4) for an hour then incubated with an increasing level of ethanol (50%, 70%, 80%, 90% then 100%). The heart was then embedded in paraffin then sectioned using microtome into (4 μM) sections. Heart sections were placed on microscope slides and fixed with 4% formaldehyde for 30 minutes. Slides were then treated, according to the manufacturer’s instruction, with TNF-α kit (MaxTag™ Histo IHC Kit, Rockland-inc, USA).

Photography & immunohistochemically staining analysis of sections Aperio positive pixel count algorithms program from Aperio image scope software v12.1.0.5029 (Aperio Technologies Inc, USA) used for digital heart slides analysis. These algorithms allow to quantify TNF-α marker within the sections to automatically calculate the prevalence of TNF-α in each section. This programme calculates the number of pixels which have stained with the kit’s dye and calculate the average intensity of staining for that dye across the tissue section.

Statistical analysis Data were presented as mean ± SEM and analysed by one-way ANOVA followed by Newman Keuls post-hoc test using GraphPad Prism® software (2007, version 5.01, USA). At p value < 0.05, differences considered as significant

3. Results

Effect of metformin on serum BNP level Isoproterenol induced a significant increase (p<0.05 compared with saline treated rats) in the serum level of BNP. In diabetic rats, metformin (75, 150 and 300 mg/kg) significantly attenuated (p<0.05) isoproterenol-induced elevated BNP level by (16%, 26%, and 41%, respectively) compared to the vehicle group (Figure 2). Similarly, metformin treatment (150 and 300 mg/kg) effectively protected non-diabetic heart as evidence of the mitigated level of BNP (p<0.05) by (21%, 36% respectively) compared to the control (Figure 3).

![Figure 1: Schematic diagram showing the experiment protocols to induced diabetes and cardiac injury.](image1)

![Figure 2: Serum brain natriuretic peptide (BNP) levels for diabetic groups. Data were analysed using one-way ANOVA followed by Newman Keuls post hoc test and reported as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs control (saline). Rats treated with Metformin 75, 150, and 300 mg/kg/24hrs i.p. (n = 8). Data expressed as a Mean ± SEM.](image2)
Metformin treatment protected the heart against isoproterenol by abrogated the elevated level of cTnT in diabetic rat in a dose dependent manner with maximum protection at 300 mg/kg/day compared to vehicle-treated diabetic rat (p<0.001, Figure 6). Similarly, metformin therapy (150 and 300 mg/kg/day) for 6 weeks before isoproterenol attenuated level of serum cTnT significantly (p<0.05) by (23% and 36%, respectively) compared to control group in normoglycemic rats (Figure 7).

**Effect of metformin on serum MMPs-1 level**

In diabetic groups, all three dosage schedules of metformin (75, 150, and 300 mg/kg/day) suppressed the elevated level of MMP-1 post isoproterenol administration significantly (p<0.05) by (16%, 29%, and 40% respectively) compared to saline-treated group (Figure 4). In the absence of diabetes, metformin treatment at (150 and 300 mg/kg/day) for 6 weeks prior to isoproterenol-induced myocardial infarction significantly abrogated the elevated level of MMP-1 (p<0.05) by (22% and 30% respectively) compared to control group (19.83 ± 0.717 ng/L, p<0.001, Figure 5).

**Fig 3: Serum brain natriuretic peptide (BNP) levels for non-diabetic groups. Data were analysed using one-way ANOVA followed by Newman Keuls post hoc test and reported as mean ± SEM. *p<0.05, ***p<0.001 vs control (saline). Rats treated with Metformin 75, 150, and 300 mg/kg/24hrs i.p. (n = 8). Data expressed as a Mean ± SEM.**

**Effect of metformin on serum cTn-T level**

**Metformin treatment protected the heart against isoproterenol by abrogated the elevated level of cTn-T in diabetic rat in a dose dependent manner with maximum protection at 300 mg/kg/day compared to vehicle-treated diabetic rat (p<0.001, Figure 6). Similarly, metformin therapy (150 and 300 mg/kg/day) for 6 weeks before isoproterenol attenuated level of serum cTn-T significantly (p<0.05) by (23% and 36%, respectively) compared to control group in normoglycemic rats (Figure 7).**

**Fig 4: Serum matrix metalloproteinase-1 (MMP-1) levels for diabetic groups. Data were analysed using one-way ANOVA followed by Newman Keuls post hoc test and reported as mean ± SEM. *p<0.05, ***p<0.001 vs control (saline). Rats treated with Metformin 75, 150, and 300 mg/kg/24hrs i.p. (n = 8). Data expressed as a Mean ± SEM.**

**Fig 5: Serum matrix metalloproteinase-1 (MMP-1) levels for non-diabetic groups. Data were analysed using one-way ANOVA followed by Newman Keuls post hoc test and reported as mean ± SEM. *p<0.01, ***p<0.001 vs control (saline). Rats treated with Metformin 75, 150, and 300 mg/kg/24hrs i.p. (n = 8). Data expressed as a Mean ± SEM.**

**Fig 6: Serum cardiac troponin-T(cTn-T) levels for diabetic groups. Data were analysed using one-way ANOVA followed by Newman Keuls post hoc test and reported as mean ± SEM. *p<0.05, ***p<0.001 vs control (saline). Rats treated with Metformin 75, 150, and 300 mg/kg/24hrs i.p. (n = 8). Data expressed as a Mean ± SEM.**

**Fig 7: Serum cardiac troponin-T(cTn-T) levels for non-diabetic groups. Data were analysed using one-way ANOVA followed by Newman Keuls post hoc test and reported as mean ± SEM. *p<0.05, ***p<0.001 vs control (saline). Rats treated with Metformin 75, 150, and 300 mg/kg/24hrs i.p. (n = 8). Data expressed as a Mean ± SEM.**

**Effect of metformin treatment on the expression of TNF-α**

TNF-α is a potent pro-inflammatory mediator and can trigger different forms of cytokines and inflammatory mediators. Metformin (150 and 300 mg/kg) significantly suppressed the expression of TNF-α in cardiac tissue (p<0.05) by (63%, and 84%, respectively) compared with vehicle-treated diabetic rats (Figure 8). In non-diabetic groups, metformin with doses (150 and 300 mg/kg) suppressed the expression of TNF-α in cardiac tissue significantly (p<0.05) by (64% and 83%, respectively) compared with the control group (Figure 9).

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Failure [17]. The level of BNP usually increases when there is a stress or abnormality in the heart chamber or in volume overload. Drugs that inhibit the level of BNP have a cardioprotective effect as its reflect the positive drug effect on the left ventricle, MI, HF [18]. In diabetic patients, BNP level was elevated as a result of volume expansion and fluid overload while glycemic control led to a significant decrease in the BNP level [19]. Isoproterenol caused endoplasmic reticulum (ER) stress and elevation of BNP level through AMPK inhibition compared with normal non-treated rats [16]. It has been reported that metformin exerts its cardioprotective effects through activation of AMPK, inhibition of ER stress and attenuation of elevated BNP level. In line, metformin treatment for 4 weeks (100 mg/kg/24hr) produced a significant reduction (p<0.05) in the BNP level compared with non-treated rats [16]. Furthermore, metformin could also signal via enhancing the activating of other cardioprotective kinases. For example, metformin elicited its cardioprotection through enhancing the phosphorylation of eNOS at Ser1177 and upregulated eNOS mRNA expression. Nitric oxide mediates crucial physiological actions, including inhibition of oxidative stress, platelet aggregation, vasodilation, leucocyte chemotaxis and apoptosis, which make it a potent cardioprotective signalling molecule [20]. Clinically, it has recently been shown by Hamasaki and colleagues that metformin treatment for 90 days significantly mitigated the level of BNP in type-2 diabetic patients. Likewise, four months treatment with metformin significantly reduced the BNP level in heart failure patients compared to placebo-treated patients [21]. These findings are in line with the above results showing that metformin can protect the heart against cardiac injury in the presence and absence of diabetes mellitus.

This study shows for the first time a head-to-head comparison for the cardioprotective of metformin against isoproterenol-induced myocardial infarction diabetic and non-diabetic rats in vivo.

The finding support that hypothesis that metformin has cardioprotective activities and it is signaling through a mechanism which is functioning in the presence somebodies such as type-2 diabetes mellitus.

Catecholamine, in general, may go through auto-oxidation pathway which result in the modulation of oxidative products and generation of oxygen reactive species (ROS). The ROS is one of the main determines to the outcome of myocardial infarction. Those oxidative products are believed to be responsible for myocardial damage, however, the exact mechanism by which isoproterenol induces myocardial infarction and cardiomyopathy is still under investigation and yet to be defined. It has been suggested that isoproterenol could induce imbalance between oxygen demand and supply because of the enhanced myocardial activity [15]. Accordingly, the energy depletion th uscan cause a cascade of biochemical and structural changes leads to myocardial cell damage. The use of isoproterenol-induced myocardial infarction is considered as a relatively easy technique, reproducible and required only a short time to induce myocardial infarction compared to coronary occlusion technique [16].

The BNP is a neuro-hormone, released mainly from the left ventricle which plays an important role in the regulation of body fluids and blood pressure and it is an early indicator for acute and chronic heart against cardiac injury in the presence and absence of diabetes mellitus.

MMP-1 is a protease which is involved in tissue remodeling in physiological and pathological conditions. In cardiac pathology, there is an imbalance between tissue-derived inhibitor of metalloproteinase (TIMP) and MMP which leads to excessive activation of MMP. Elevated level of MMP-1 then stimulate abnormal cardiac extracellular matrix (cECM) expression, leading to vascular and cardiac abnormalities [18]. Recent studies showed that MMP-1 activity was up-regulated in cardiac tissues by β-adrenergic stimulation in vivo. These results using a MMP inhibitor suggest that both MMPs may participate in the myocyte hypertrophy induced by β-adrenergic stimulation through the IGF axis [22]. The MMP plays an important role in the myocardial dysfunction following ischaemia/reperfusion injury in rat [18]. The early increase in MMP activity produces a proteolytic environment that may contribute to myocardial stunning injury in humans [23]. Metformin exerts a cardioprotective effect through influencing PKC-dependent activation of MMP-1 by inhibition of ERK1/2/NF-κappaB pathway in human aortic smooth muscle cell [24]. In addition, the glycolytic control achieved by metformin in diabetic rats is reported to protect the vasculature against hyperglycinemia-induced insults and mitigate vascular remodeling [25]. Metformin treatment (150 mg/kg/24hr) significantly reduced the level of MMPs in isoproterenol-induced cardiac hypertrophy in mice [26]. Moreover, it has been reported that metformin treatment (300 mg/kg) for 4 weeks produced a significant reduction in MMP activity in insulin-resistant rats compared with non-treated rats [27].
results of this study are in line with others and show that metformin treatment for 6 weeks produced a dose dependent suppression in the serum MMP-1 level in both diabetic and non-diabetic rats.

Cardiac Tn-T is a tissue specific biomarker for myocardial injury and extensively used clinically. It has been demonstrated that a bolus dose of metformin (125 µg/kg) before ischaemia or at reperfusion significantly reduced myocardial injury and limited cTn-T level following myocardial ischaemia/reperfusion injury in nondiabetic and diabetic mice[6]. Chronic use of metformin in diabetic patient with ST-segment elevation myocardial infarction exhibited a significant attenuation in cTn-T level compared with non-treated patients [28]. Furthermore, metformin abrogated the elevated level of cTn-T induced in isoproterenol-induced myocardial infarction in rat 29. Taken together, the results of this study are consistent with others and demonstrated that metformin treatment produced a dose dependence suppression in the elevated level of cTn-T in both diabetic and non-diabetic rats.

TNF-α is a pro-inflammatory cytokine, produced from nucleated cells in the heart. The level of TNF-α is elevated in case of heart failure. The inflammatory response following an ischaemic event in the heart triggers innate stress kinases and cytokines which exacerbate the cardiac damage and affect deteriorate cardiac contractility30. Using Human umbilical vein endothelial cells, it has been reported that a unique anti-inflammatory effect of metformin when it reversed the deleterious effects of TNF-α via PI3K/AKT dependent signaling mechanism31. Chronic treatment with metformin significantly protected the heart and suppressed the level of TNF-α in metformin-treated rats [32,34]. Moreover, it has also been shown that metformin treatment suppressed the TNF-α through activation of AMPK in rats [35,36]. Furthermore, acute and chronic treatment with metformin exerted a dose dependant reduction in TNF-α through activation of AMPK [37,38]. Together, the data of this study are showing that metformin possesses a considerable anti-inflammatory effect which could have a significant clinical implication in diabetes and other pathologies.

Conclusion

The principle findings of this study are; metformin treatment limited myocardial infarction when it was given before cardiac injury, and, the cardioprotective mechanism of action of metformin functions in the presence and absence of diabetes mellitus.

This study provides a convincing evidence that metformin has cardioprotective effect which can potentially protect the heart against myocardial infarction and improve the clinical outcome. Cardioprotection established by metformin mitigated tissue injury biomarkers following isoproterenol in non-diabetic and diabetic rats. Characterisation of the signalling pathway by which metformin mediates its cardioprotective could be an interesting topic for future investigation.

Conflict of interest

There is no conflict of interest.

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