Original Article

Myocardial Salvaging Effect of Metformin In Isoproterenol Induced Myocardial Injury in Rats

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Abstract

Background: Cardiovascular disease is one of the most common causes of death. Various clinical and experimental investigations demonstrate that metformin, a widely used anti-diabetic drug, exhibits cardioprotective properties against myocardial infarction. The present study was designed to observe the cardioprotective effect of metformin, in isoproterenol induced myocardial injury in rats.

Methods and Materials: A total number of 35 rats were taken for the experiment. Wistar Albino rats of either sex, were divided into 5 groups (n=7). After grouping, two groups Group III and Group V were induced diabetes by administration of single dose of Alloxan Monohydrate 120mg/kg body weight intraperitoneally. Group IV and V received metformin 100mg/kg/day for 4 weeks (2nd week – 5th week) and then isoproterenol 85mg/kg/day was given intraperitoneally 24 to 48 hour prior to scarifice (1st and 2nd day of 6th week). Biochemical parameters LDH and troponin I levels were estimated. Histopathological examination of heart was also done.

Results: Results showed, mean LDH, Troponin-I levels of Isoproterenol treated diabetic group G-III were 913.71±24.2, 2.19±0.07. The levels were 498.57±15.1, 1.13±0.08, in G-V. Mean LDH, Troponin-I levels were 793.71±70.4, 1.36±0.05 G–II and levels were 411.57±21.9, 0.16±0.05 in G-IV. P (<0.05) value indicates there is significant difference between G III vs G V and G II vs G IV. Microscopic examination of heart showed myocardial structure disorganization, necrosis, edema scoring "3-4" in isoproterenol treated group G II and G III. In G IV and G V microscopic examination showed reduction of necrosis and edema, nearly normal cardiac architecture scoring "0-1.

Conclusion: Study results showed that metformin has cardioprotective effect in isoproterenol induced myocardial injury.

Introduction

Cardiovascular Disease (CVD) is a global health problem with high mortality and morbidity rate [1]. Populations most affected are from low and middle income countries like Bangladesh, where 80% of these deaths occur [2]. Study from rural Bangladesh demonstrated dramatic increase in CVD, and the age-standardized CVD mortality has increased by 30-fold (from 16 deaths per 100,000 to 483 per 100,000) among males and 47-fold (from 7 deaths per 100,000 to 330 deaths per 100,000) in females (1976-2016) [3].

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Isoproterenol induced myocardial infarction serves as a standardized model as pathophysiological changes in heart muscle of experimental animal, similar to that observed in human myocardial infarction. Several mechanisms explained isoproterenol induced myocardial injury. There is an imbalance between oxygen supply to and demand from cardiomyocytes , which is related to myocardial hyperfunction due to increase both in chronotropism and inotropism. There is also an elevation of Ca++ overcharge inside the cell and this is related to the activation of the adenylatecyclase enzyme and the depletion of ATP levels [4]. Isoproterenol generates free radicals which initiates peroxidation of membrane bound polyunsaturated fatty acids leading to damage of the integrity of myocardium. Myocyte death causes cytosolic contents to diffuse to the systemic circulation. Thus causes elevation of serum activity of cardiac biomarkers [5].

Diabetes mellitus increases the risk of developing CVD by approximately 5-fold and the risk of heart failure and death after a myocardial infarction upto 4 fold [6]. Metformin, an oral anti-diabetic drug from the biguanide, insulin sensitizing class, is primarily used in the management of type 2 diabetes mellitus[7]. It is the only therapeutic agent that has been demonstrated to reduce the macrovascular events[8]. It proves that the cardioprotective effects of metformin are not solely due its anti-hyperglycemic properties [9].

AMPK is a major regulator of cellular energy and is activated by an increase in the cellular AMP: ATP ratio during energy stress. AMPK was shown to maintain the energy balance in cells during ischemia by increasing ATP levels. Metformin increases AMPK activation in both ischemic and non-ischemic hearts. Different studies showed that rats treated with metformin showed a significant reduction of infarct size compared with untreated animals. For evaluation of cardioprotective effect of metformin, an experimental study design is possible to replicate the occurrence of myocardial injury in experimental animals, by the administration of cardio-toxic drugs, like catecholamines in high doses [8].

The purpose of the study is to evaluate the cardio-protective effect of metformin in isoproterenol induced myocardial injury in rats.

Materials and methods
The study was carried out in the Department of Pharmacology and Therapeutics of Sir Salimullah Medical College and Mitford Hospital (SSMC), Dhaka in collaboration with Institute of Nutrition and Food Sciences (INFS), University of Dhaka. The total study period was from July 2018 to June 2019.

Study population: A total number of 35 healthy adult Wistar Albino rats of both sex, weighing approximately 120 to 135 grams, 10-12 weeks of age were purchased from animal house of Department of Pharmacy, Jahangirnagar University, Savar, Dhaka, Bangladesh. The rats were acclimatized in metallic cage in the animal house of Institute of Nutrition and Food Sciences at University of Dhaka for 2 weeks before the actual experiment.

The environment of the animal house was properly maintained which was essential for wellbeing of the animals. Rat diet was placed in the cages. Each cage was properly labelled for identification of different groups

After acclimatization, 35 rats were grouped into 5 groups, each group containing 7 rats and experimental rats of different groups were kept in different cages.

Drugs used:
Tab Metformin (500mg) was obtained from Square pharmaceuticals, Bangladesh. **Alloxan Monohydrate** was supplied by Institution of Nutrition and Food Sciences, University of Dhaka. Alloxan Monohydrate was dissolved in sterile normal saline and administered by single intra peritoneal injection at a dose of 120mg/kg of body weight. **Inj. Isoproterenol** was procured from Samarth Pharma Life Sciences, India. Inj. Isoproterenol was given intra peritoneally at a dose of 85 mg/kg of body weight.

Experimental schedule:
The rats were divided into 5 groups, containing 7 rats in each group after acclimatization. Among the 5 groups, two groups (group III and group V) were kept overnight fasting after acclimatization and then 120 mg/kg body weight of Alloxan Monohydrate [10] was induced intraperitoneally to each of the rats of group III and group V. After 72 hours of alloxan injection, blood from animals were taken and serum blood glucose were estimated. Blood glucose level >200mg/dl was considered as diabetes mellitus[11]. The experimental 5 groups were arranged as follows-
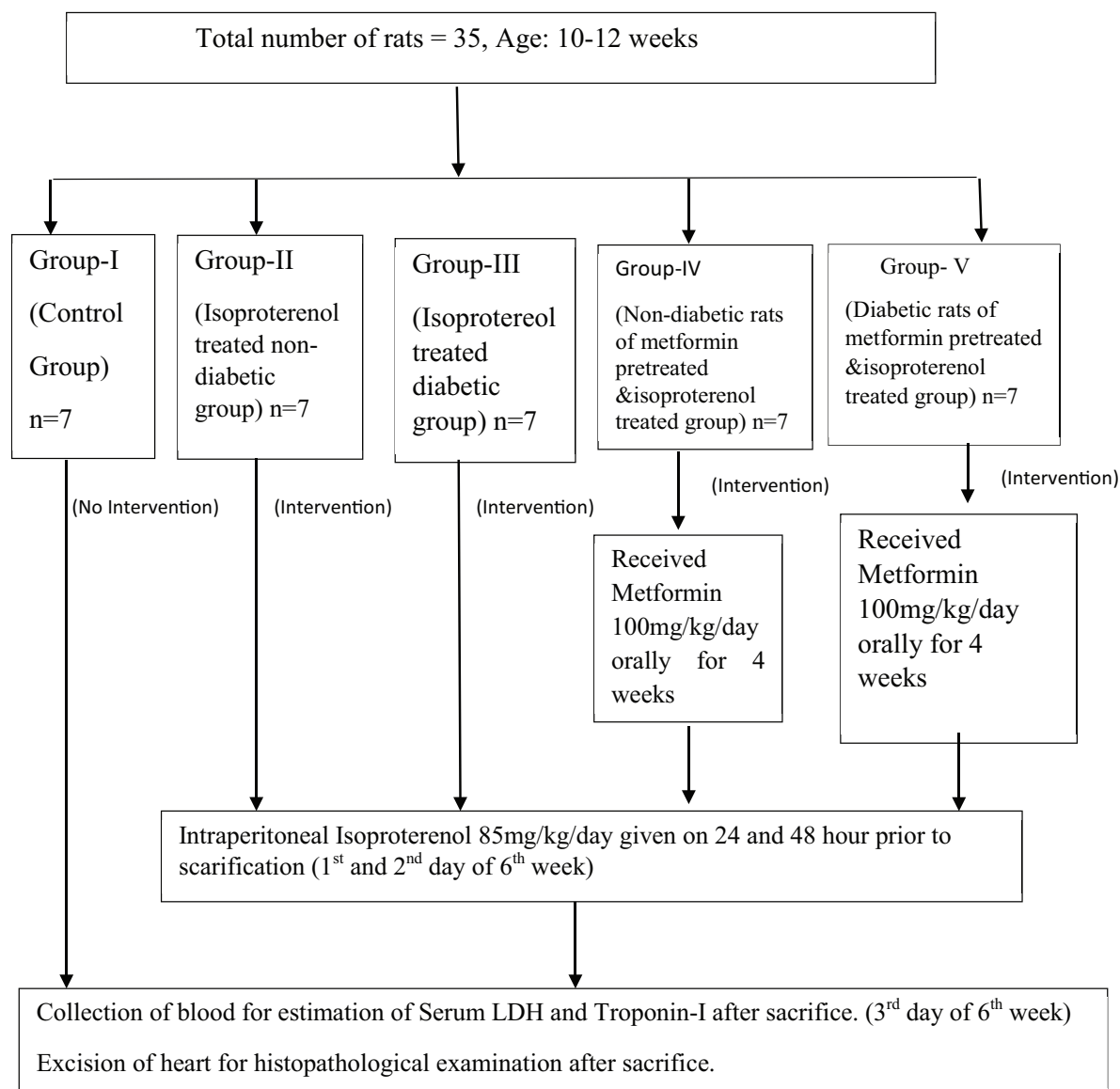
Group-I (Control group):-This group of animals were given normal lab diet upto sacrifice.

Group-II (Isoproterenol treated non-diabetic group):-This group of animals received lab diet upto sacrifice. Myocardial infarction was produced by Isoproterenol (85mg/kg body weight) injection intraperitoneally 24 and 48 hour prior to scarifice (1st and 2nd day of 6th week) [11].

Group-III (Isoproterenol treated diabetic group): The Alloxan Monohydrate (120 mg/kg body weight) was injected intraperitoneally to induce diabetes at 1st week (day 1) and administered with Isoproterenol (85mg/kg body weight) injection intraperitoneally 24 to 48 h prior to scarifice (1st and 2nd day of 6th week).

Group-IV (Non-diabetic rats of metformin pre-treated and isoproterenol treated rats):- This non-diabetic group of rats received metformin (100mg/kg/day, orally) from 2nd week to 5th week (4 weeks). Myocardial infarction was produced by Isoproterenol (85mg/kg body weight) injection intraperitoneally 24 and 48 hour prior to sacrifice (1st and 2nd day of 6th week).

Group-V (Diabetic rats of metformin pre-treated and isoproterenol treated group):- Alloxan Monohydrate (120 mg/kg body weight) was injected intraperitoneally to induce diabetes mellitus at 1st week (day-1). Metformin (100mg/kg/day) was fed orally from 2nd week to 5th week (4weeks). The rats were given with Isoproterenol

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Total number of rats = 35, Age: 10-12 weeks

| Group-I (Control Group) n=7 | Group-II (Isoproterenol treated non-diabetic group) n=7 | Group-III (Isoprotereol treated diabetic group) n=7 | Group-IV (Non-diabetic rats of metformin pretreated &isoproterenol treated group) n=7 | Group- V (Diabetic rats of metformin pretreated &isoproterenol treated group) n=7 |

(No Intervention)   (Intervention)   (Intervention)   (Intervention)   (Intervention)

Received Metformin 100mg/kg/day orally for 4 weeks

Received Metformin 100mg/kg/day orally for 4 weeks

Intraperitoneal Isoproterenol 85mg/kg/day given on 24 and 48 hour prior to scarification (1st and 2nd day of 6th week)

Collection of blood for estimation of Serum LDH and Troponin-I after sacrifice. (3rd day of 6th week)

Excision of heart for histopathological examination after sacrifice.

(85mg/kg body weight) injection intraperitoneally 24 and 48 hour prior to sacrifice (1st and 2nd day of 6th week).

Induction of diabetes mellitus:

Alloxan monohydrate (234mg) was dissolved in 3 ml of Normal saline. A light violet colour clear solution was made in a vial. The drug was administered by single intraperitoneal injection at a dose of 120mg/kg of body weight (200 micro lit/ rat) in 14 rats of group-III and group V at 1st week (day 1).

Experimental dose of metformin:
Tablet Metformin was given at a dose of 100mg/kg [11] dissolving in distilled water along with lab diet to the group IV and group V for 2nd week to 5th week (4 week). The tablets were crushed and dissolved in distilled water and given at a volume of 200 micro lit per rat.

Induction of myocardial injury:-

Myocardial injury was induced in 28 rats (group II-V) by administration of injection isoproterenol intraperitoneally 85mg/kg/day 24 and 48 hour prior to scarifice (1st and 2nd day of 6th week).

Study parameters:

Serum lactate dehydrogenase was estimated by UV enzymatic method (Kinetic method). Serum Troponin-I was estimated by Immunofluorescence Assay.

Sacrifice of animals and collection of blood:

After completion of experiment, the rats were anesthetized by chloroform and then sacrificed (3rd day of 6th week)[11]. Approximately 3 to 4 ml of blood were collected from each rat and were taken in separate test tubes with proper identification numbers. The blood samples were allowed to clot and the serum was separated by centrifugation and collected. After collection, it was transferred to separate eppendorf tubes. Then the serum was kept in refrigerator at -27°c and taken to Biochemistry department of SSMC to analyze various biochemical parameter.

Excision of hearts for Histopathology:

After collection of blood sample, chest of the animals were opened. Heart was excised. The heart was examined thoroughly by naked eyes. Then heart was preserved in 10% formalin for histopathology .This was taken to Pathology department of SSM&CH and then undergo dehydration, clearing, paraffin infiltration, embedding, section cutting and staining for histological examinations.

Statistical analysis:

The results were expressed as mean ± SD (Standard deviation). Statistical significance of differences between groups was determined by one way **ANOVA** test and **Bonferroni** test. P values of < 0.05 were considered statistically significant. The calculations were performed by using SPSS version 22.

Results

Table-I
Fasting Blood Glucose (mmol / L) level in different groups of the rats (n=35)

Groups	Fasting Blood glucose (mmol/L) Day 1	Fasting Blood glucose (mmol/L) Day 4
Group I (n=7)	4.43±0.39	4.46±0.36
GroupII (n=7)	3.87±0.42	3.97±0.32
Group III(n=7)	4.77±0.34	11.99±0.31
Group IV(n=7)	3.94±0.44	4.66±0.39
Group V(n=7)	4.80±0.24	12.00±0.24

Table II (a)
Serum LDH and Troponin-I levels in different groups of rats after experiment, 3rd day of 6th week (n=35).

Groups	Serum LDH (U/L)	Troponin-I (ng/ml)
Group-I (n=7)	234.14±22.3	0.01±0.00
Group-II (n=7)	793.71±70.4	1.36±0.05
Group-III (n=7)	913.71±24.2	2.19±0.07
GroupIV(n=7)	411.57±21.9	0.16±0.05
Group-V (n=7)	498.57±15.1	1.13±0.08
p-value	<0.001	<0.001

Results are expressed as Mean±SD. One way ANOVA followed by Bonferroni test was performed to compare between groups.

The test of significance was calculated and p values < 0.05 was accepted as level of significance.

Table II (b)

Comparison of Serum LDH and Troponin-I levels among different groups of rats after experiment, 3rd day of 6th week (n=35).

Groups	P values	
	Serum LDH	Troponin-I
I vs II	<0.001**	<0.001**
I vs III	<0.001**	<0.001**
I vs IV	<0.001**	<0.001**
I vs V	<0.001**	<0.001**
II vs III	<0.001**	<0.001**
II vs IV	<0.001**	<0.001**
II vs V	<0.001**	<0.001**
III vs IV	<0.001**	<0.001**
III vs V	<0.001**	<0.001**
IV vs V	<0.001**	<0.001**

ANOVA was used to analyze the data and comparison between two groups were done by Bonferroni test.

In case of serum LDH * indicates significant difference (p value < 0.05) between groups I vs II, I vs III, I vs IV, I vs V, II vs III, **II vs IV**,II vs V, III vs IV, **III vs V**,IV vs V.

In case of serum troponin-I *indicates significant difference (p value <0.05) between groups I vs II, I vs III, I vs IV, I vs V, II vs III, **II vs IV,** II vs V, III vs IV, **III vs V**,IV vs V.

Serum LDH level is 234.14 U/L, that is within normal range in Group-I. In group II it is 793.71 U/L that is higher than normal range and in group III it is 913.71 U/L which is highest among all the groups. In group IV it is 411.57 U/L which is within normal range and in group V it is 498.57 U/L which is above normal range. Significant difference is observed between group **II vs IV** and group **III vs V.**

Serum Troponin-I level is 0.01 ng/ ml, that is within normal range in Group-I. In group II it is 1.36 ng/ml that is higher than normal range and in group III it is 2.19 ng/ml which is highest among all the groups. In group IV it is 0.16 ng/ml which is within the normal range and in group V it is 1.13ng/ml which is above normal range. Significant difference is observed between group II vs IV and group III vs V.

In this study, histopathological examination of heart revealed score '0' in Group I. In Group II showed score "3-4" in histopathological examination.

In Group III showed score "3-4" in histopathological examination. Findings in these two groups were marked myocardial disorganization, edema and necrosis. Group IV revealed score "0-1" in histopathological examination. Group V showed score "0-1" in histopathological examination. Findings in these two groups are nearly normal cardiac architecture, scanty necrosis and partial absence of myocardial basement membrane

Table III

*Histopathological findings of heart in different groups of rats (n = 35).*

| Group	Observation	Result/findings	Score
Group I (Normal control rats)	Architecture of - Myocardium - Endocardium - Pericardium	Normal histological finding	0
Group II (Isoproterenol treated non diabetic rats)	- Myocardial disorganization - Marked loss of myocardial basement membrane - Marked oedema and necrosis present	Marked histological changes	3-4
Group III (Isoproterenol treated diabetic rats)	- Wide spread myocardial disorganization - Marked loss of myocardial basement membrane - Marked oedema and necrosis present	Marked histological changes	3-4
Group-IV (Metformin pre-treated&isoproterenol treated non-diabetic rats)	- Nearly normal cardiac architecture - Scanty necrosis - Partial absence of myocardial basement membrane	Nearly normal	0-1
Group-V (Metformin pre-treated&isoproterenol treated diabetic rats)	- Nearly normal cardiac architecture - Scanty necrosis - Partial absence of myocardial basement membrane	Nearly normal	0-1
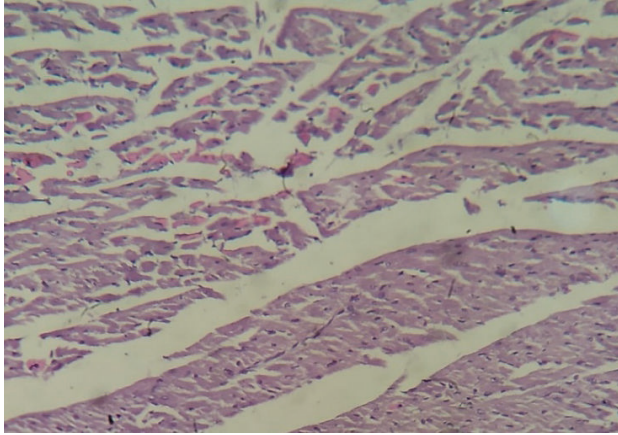
Photomicrograph 1: (Magnification at 40X objectives) showing the normal cardiac architecture (group-I)
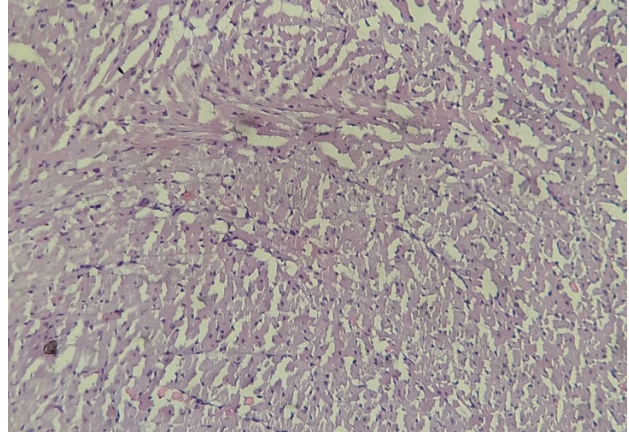

Photomicrograph 2:(Magnification at 10X objectives) showing normal architecture of myocardium (group-I)
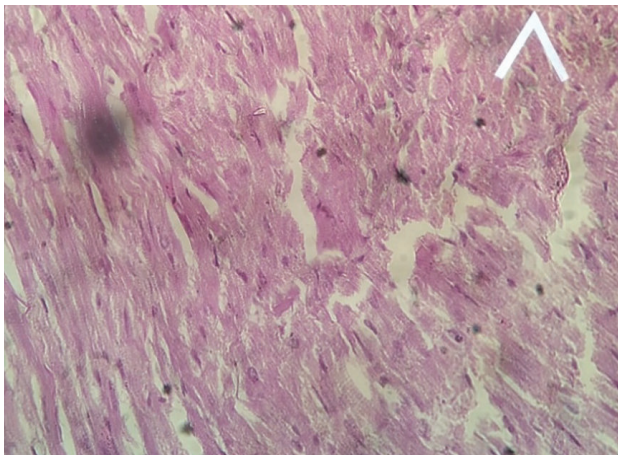

Photomicrograph 3: Degenerative changes, edema, and separation of muscle fibers in isoproterenol treated non-diabetic rats (40 X magnification) (group-II)
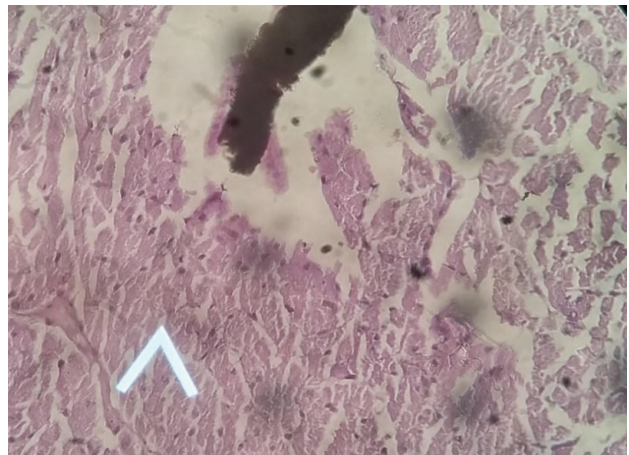
Photomicrograph 4: Degenerative changes, edema, and separation of muscle fibers in isoproterenol treated diabetic group of rats (40 X magnifications) (group-III)
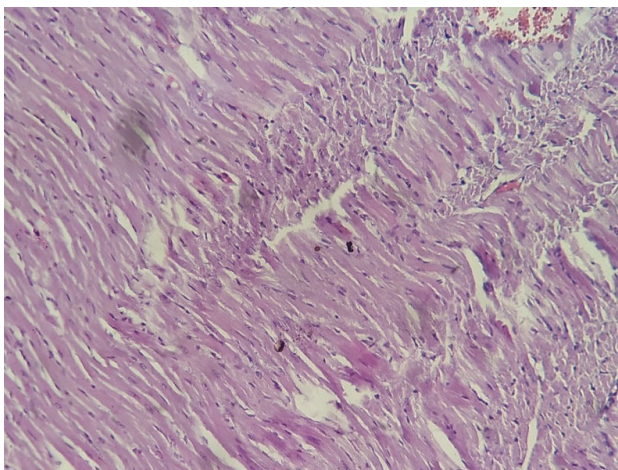
Photomicrograph 5: Reduced edema, necrosis showing in metformin pretreated and isoproterenol treated non-diabetic rats (10x magnification) (group-IV)
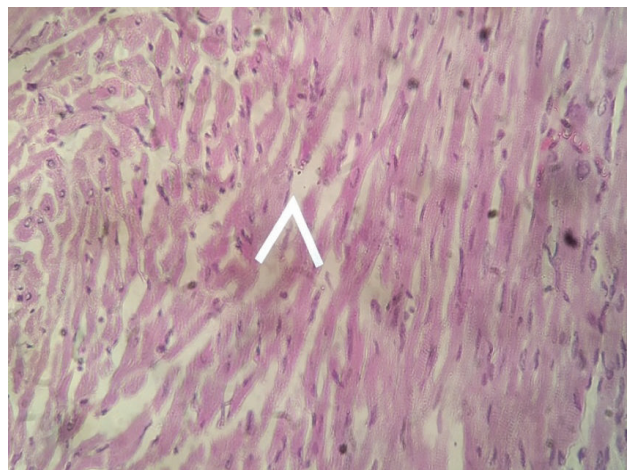
Photomicrograph 6: Reduced edema, necrosis showing in metformin pretreated and isoproterenol treated diabetic rats (40x magnification) (group-V)

Discussion

Cardiovascular disease (CVD) is a major health problem throughout the world and a common cause of morbidity and mortality [13]. The present study was carried out to evaluate the cardioprotective effect of metformin on Wistar Albino rats. As metformin is an anti-diabetic drug, here effect of metformin has been observed in both diabetic and non-diabetic cardiac injured rats [14]. Assessment of cardiac function was made by estimating serum LDH, Troponin-I and histopathological analysis of heart.

In regards to quantitative estimation of the serum LDH levels in this study, metformin was found to have a protective role on myocardium. The decrease in the serum enzyme levels reflects a reduction in the severity of myocardial injury[8.] Mean serum LDH level in group III (Isoproterenol treated diabetic group) was 913.71±24.2, in group II (Isoproterenol treated non-diabetic group) it was 793.71±70.4. In group IV (Non-diabetic rats of metformin pre-treated and isoproterenol treated group) it was 411.57±21.9 and in group V (Diabetic rats of metformin pre-treated and isoproterenol treated group) it was 498.57±15.1. p value (<0.001) shows significant difference was found in group II vs group IV and group III vs group V, which suggests that metformin possesses cardioprotective effect. Our results are in agreement with previous study done by Kiran A Bhave.[8] who showed pre-treatment with metformin 100mg/kg significantly reduced elevated serum LDH levels and protection was statistically similar to that of carvedilol.

The level of troponin-I is a highly sensitive and specific marker of myocardial cell injury. Elevated troponin I levels predict the risk of both cardiac cell death and subsequent infarction [15]. Mean serum troponin-I was 2.19±0.07 in group III (Isoproterenol treated diabetic group). It was 1.36±0.05 in group II (Isoproterenol treated non-diabetic group). It was 0.16±0.05 in group IV (Non-diabetic rats of metformin pre-treated and isoproterenol treated group). The value was 1.13±0.08 in group V (Diabetic rats of metformin pre-treated and isoproterenol treated group). p value (<0.001) shows significant difference between group II vs group IV and group III vs group V, which suggests that metformin possesses cardioprotective effect. Our results are in

agreement with previous study done by Kiran A Bhave[8] and John W. Calvert.[16] They showed that pre-treatment by metformin before cardiac injury can reduce troponin levels. John W. Calvert.[16] showed that following myocardial injury, metformin (125µg/kg) significantly (p=0.026) attenuated rise of troponin by 56% (13.63±3.12 vs 5.99±0.49ng/ml).

Histopathological examination of myocardial tissues in group I revealed clear integrity of the myocardial cell membranes. All of the rat heart showed score "0" in this group. Heart tissues from rats in group II and group III showed myocardial structure disorganization, cardiac muscle fibre separation, necrosis, edema scoring "3-4". The histopathological findings of the metformin pre-treated myocardial infarcted hearts showed nearly normal cardiac tissue in group IV and group V scoring "0-1". The reduced cardiac muscle fiber, architectural damage and necrosis in group IV and group V confirmed the cardioprotective effect of metformin. These observations of the present study coincide with previous similar study done by Yahya G. Karwi[12] (metformin 150mg/kg pre-treated cardiac tissue showed moderate myocyte necrosis), Manjusha K. Borde.[11] (metformin 100mg/kg pre-treated cardiac tissue showed occasional edema and necrosis) Whittington et al.[14] and Soraya et al.[9]

Results of the study showed pre-treatment with metformin significantly reduced cardiac biomarkers associated with ISO-induced myocardial injury. These findings were confirmed by histopathological examination. So, metformin possesses cardioprotective effect in myocardial injury.

Summary and conclusion

From the observations of the study, it shows that pre-treatment by metformin, prior to a subsequent isoproterenol induced myocardial injury, demonstrates a cardioprotective effect. This observation may have clinical relevance, as it can be developed as a new therapeutic approach for cardiovascular risk prevention.

Limitations of the study
- Sample size was small.
- Duration of study was short.
- Expensive.

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