Original Article

Evaluation of Cardioprotective Effect of Metformin in Isoproterenol Induced Myocardial Injury in Rats

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Abstract

Cardiovascular disease is one of the common causes of death worldwide, thus, there is an increasing need for discovering and identifying various measures for cardioprotection.

This study evaluated the hypothesized cardioprotective effect of metformin therapy, in isoproterenol induced myocardial injury in rats.

Normoglycemic Wistar rats of either sex, were divided in 4 groups (n=8) each.Two test groups were administered two different doses of metformin (100 mg/kg/day and 225 mg/kg/day respectively) orally, for 7 days prior to myocardial injury. Carvedilol (10 mg/kg/day by oral gavage for 7 days) was administered to the positive control group. Evaluation parameters were 1. Pathological-(*Histopathological grading* and*Heart coefficient*) & 2. Biochemical - (serum *cardiac Troponin T* and *lactate dehydrogenase*).

The findings of our study are suggestive of cardioprotection, offered by prior short-term metformin therapy, against a subsequent myocardial insult, in normoglycemic experimental animals. This is a significant observationfor cardiovascular risk prevention in a clinical setting.

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Introduction

Cardiovascular disease is now the most common cause of death worldwide, accounting for about 30% of the human deaths, including nearly 40% in high-income countries and about 28% in low- and middle-

income countries (1). The burden of cardiovascular mortality and morbidity has been growing in significant proportions, and thus, the need for discovering and identifying various measures for cardioprotection.

Metformin, an oral medication belonging to the biguanide group is primarily used in the management of Type II Diabetes Mellitus, particularly in overweight and obese patients. It has an anti-hyperglycemic action, as it reduces the elevated blood glucose levels in diabetic patients. In the management of Type II Diabetes Mellitus, it has been observed that glycemia control (reduction in the Hemoglobin A_{1C} levels) by any therapy (insulin or oral agents) diminishes the microvascular complications of the disease. Metformin, however, is the only therapeutic agent that has been demonstrated to reduce the macrovascular events (2).

Metformin therapy has demonstrated a greater reduction in the risk for any diabetes related end-point (including macrovascular as well as microvascular complications), all-cause mortality and stroke, as compared to insulin or sulfonylurea therapy. As an equivalent glycemia control (HbA_{1C} levels) was achieved with either the metformin or sulfonylurea/insulin therapy, the additional beneficial effects observed with metformin therapy, predominantly on the cardiovascular outcomes, could not be explained by glycemia control (3).

Thus, metformin therapy may be hypothesized to possess a cardioprotective activity, but this hypothesis remains to be conclusively proven. A substantiation of this hypothesis may be possible, if cardioprotection may be demonstrated in non-hyperglycemic subjects. This is because metformin demonstrates an antihyperglycemic effect rather thana hypoglycemic effect (2). Hence, metformin therapy would not be expected to lower the blood glucose levels in subjects with normal baseline blood glucose levels. Thus, a study carried out in normoglycemic subjects, would be an appropriate approach for the evaluation of the hypothesized direct cardioprotective effect of metformin therapy.

For such an evaluation, an experimental study design, using an appropriate translational animal model for the demonstration of cardioprotection, may have an advantage. This is because a precise evaluation is possible under the controlled experimental conditions, in a laboratory. It is possible to replicate the occurrence of myocardial injury in experimental animals, by the administration of cardio-toxic compounds, like catecholamines in high doses (4). Hence, it is easier to predict the occurrence of a cardiac event like myocardial infarction in animal studies, and thus, to analyze the cardioprotective effect of the drug under evaluation.

The purpose of this study, is to evaluate the hypothesized cardioprotective effect of metformin therapy, using an experimental model of isoproterenol (a sympathomimetic) induced myocardial injury. If the hypothesis is proved to be correct, it would identify cardioprotection with metformin therapy. This may be useful in identifying the possible newer indications of metformin, for the patients placed at a high risk for cardiovascular morbidity.

Methods

The study methodology, including all the procedures, was approved by the Institutional Animal Ethics Committee prior to commencement. The study was an assessor blind, four-arm, parallel study design.

Animals and Diet

Wistar rats of either sex, aged between 8-10 weeks and weighing between 150-200 grams were procured from Bharat Serums and Vaccines Limited, Thane. They were managed as per the C.P.C.S.E.A. (Committee for the Purpose of Control and Supervision of Experiments on Animals) animal care guidelines, Ministry of Animal Welfare Division, Government of India. The animals were acclimatized for a period of one week prior to the start of study.

The animals were maintained on a standard pellet diet (Amrut laboratory animal feed, Nav Maharashtra Chakan Oil mills limited; energy 3620 Kcal/kg, crude protein 22.13%, crude oil 4.11%, fibre 3.43%, ash 5.3% and sand silica 1.13%). They were allowed to eat ad libitum. After a period of acclimatization for

one week in the central animal house, the animals were screened for the blood glucose levels prior to randomization to the study groups. After screening, the animals were randomly divided into 4 groups of 8 animals each: Group I (control); Group II (Positive control); Group III (Test, low dose); Group IV (test, high dose).

The study animals were treated over 10 days. The different study groups were administered the respective compounds for a period of 7 days (Day 1 to Day 7), based on the model described by Rona et al (5, 35-37). Carvedilol 10 mg/kg/day orally was selected for administration to the positive control study group (6). Carvedilol was orally administered in the form of a suspension, using carboxy-methylcellulose (CMC) as the suspending agent, as it is insoluble in the commonly used solvents (7). The calculated dose, per animal, was administered in 0.5-1 ml of the suspension (2 mg/ml), by oral gavage method.

The maximum recommended oral dose of metformin in human beings (with normal renal function) varies as 2 g, 2.5 g, 2.55 g per day, depending on the factors like control of hyperglycemia, concurrent therapy, age, etc (2, 8, 9). For our study, the high human dose of 2.5 g/day, extrapolated to the rats on body surface area basis, was selected as the dose (225 mg/kg/day) for the high-dose test group (Group IV). A dose, less than half of the high dose for the study, 100 mg/kg/day orally, was selected as the low dose for administration to the study group III (Low-Dose Test group). Freshly prepared solutions of metformin in water were administered to the test groups III and IV, by oral gavage.

The weights of the animals were followed up during the period of administration of the respective compounds. The weights on day 1 signified the weights before the administration of any compound, and the weights on day 8 signified the weights after the administration of the last dose of the respective compounds on day 7.

Myocardial injury induction was done with isoproterenol administration over the next 2 days. Isoproterenol, in a dose of 85 mg/kg/day subcutaneously, on 2 consecutive days, was selected for the induction of myocardial injury (10). A freshly prepared solution of isoproterenol in water, in a concentration of 20 mg/ml, was administered by a subcutaneous injection on the loose skin at the upper back, between the shoulders.

The animals were weighed on day 10, by a resident doctor of Pharmacology, blinded to the study groups. Forty-eight hours after the first isoproterenol dose, the animals were sacrificed by deep ether anesthesia, using 5 ml of anesthetic ether, followed by terminal blood collection from the dorsal aorta. 4-5 ml of blood was collected, of which about 1 ml of blood was heparinised and the remaining collected in a plain bulb. The heart was then exposed, separated by blunt dissection and removed. It was gently squeezed and blotted dry for weighing and immediately preserved in 10% buffered formalin for histopathological evaluation.

Parameters for Evaluation

The primary parameter for evaluation was the 'Histopathological grading' of myocardial injury, and the secondary parameters for evaluation included the 'Heart coefficient', 'Serum Cardiac Troponin T' and 'Serum Lactate Dehydrogenase levels'. The combination of biochemical and pathological parameters, as used in our study, formed a good combination for the evaluation of myocardial injury.

For the different evaluation parameters, cardioprotection was identified in terms of statistically significant reductions in the degree of myocardial injury, as compared to the disease control group, for the respective parameters.

Biochemical Parameters

Serum Cardiac-Troponin T elevation was estimated in a qualitative manner, using 'Roche Trop T Sensitive' test kit (Trop T test) (11). Qualitative detection of Troponin T elevation identified either the presence or absence of myocardial injury. Trop T test gives a positive result when the serum levels of cardiac-Troponin T are ≥ 0.08 ng/ml. Serum Lactate

Dehydrogenase (LDH) levels were estimated by the Modified IFCC (International Federation of Clinical Chemistry) method, using commercially available LDH (P-L) test-kit from Coral Clinical Systems (12), and the Fully Automated Olympus AU - 400 autoanalyser.

The quantitative estimation of the serum lactate dehydrogenase levels, combined with the qualitative estimation (raised/normal) of serum cardiac Troponin T, forms an appropriate and adequate combination for the biochemical evaluation of myocardial injury.

Pathological evaluation parameters

'Heart:Body weight ratio', also referred to as 'Heart coefficient' (13, 14) is a parameter for this experimental model, as the weight of the heart, damaged due to isoproterenol, is expected to increase due to congestion, edema and inflammatory response (15). This can be assessed by dividing the heart weight (mg) of the study animal, by the respective body weight on Day 10 (g). The heart coefficient of an adult laboratory rat is 4 mg/g (SP). 'Histopathological grading' of myocardial injury, provided for a direct observation and assessment of myocardial damage. This was based on the experimental model of isoproterenol induced myocardial injury, as described by Rona et al (5, 35-37).

GRADE	HISTOPATHOLOGICAL FINDINGS
0	No Change
1	Focal Interstitial Response
2	Focal Lesions in many sections, consisting og mottled staining& fragmentation of muscle fibers
3	Confluent retrogressive lesions with hyaline necrosis & fragmentation of muscle fibers & sequestrating mucoidoedema
4	Massive infarct with occasionally acute aneurysm &mural thrombi

Morphological changes, observable in this experimental model, include interstitial edema, subendocardial hemorrhages and congestion, myofibrillar degeneration, interstitial inflammatory response, formation of contraction bands and fibrosis.

Statistical analysis

All the statistical analyses have been carried out using 'GraphPad Prism, version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com, except, Biserial correlation coefficient (r_b), which was calculated manually using the formula and Fisher Freeman Halton test, which was analyzed using StatsDirect statistical software, version 2.7.8 (3/15/2010).

Histopathological grading was expressed in the form of median with interquartile range. Trop T test results were expressed as absolute numbers. All other quantitative data was expressed in the form of Mean±Standard deviation. In all analyses, two-tailed tests for the determination of p value were used. p-values of < 0.05 were considered to be statistically significant.

The change of body weight from Day 1 to Day 8, between the various study groups, was analyzed using One Way Analysis of Variance. Trop T test results of the various study groups, being a nominal data, were analyzed by contingency analysis using the Fisher Freeman Halton test. Serum Lactate Dehydrogenase levels and the Heart coefficient readings of the various study groups, being quantitative data, were assessed for normality using the Shapiro-Wilk test, followed by comparison using One Way Analysis of Variance with Tukey's post test.

Correlation between Trop T test results and serum Lactate Dehydrogenase levels was analyzed using the 'Biserial' correlation coefficient (r_b).

Histopathological grading, being an ordinal data, was expressed in the form of median with interquartile range for the study groups, and the medians were compared by Kruskal Wallis test followed by Dunn's Multiple Comparison Test as post test.

Results

32 Wistar rats (12 male and 20 female) were randomly assigned to the study groups. The mean blood glucose level was 101.375 mg/dl, with a standard deviation of 8.78 mg/dl.

The mean change of body weights between Day 8 and Day 1, between the various study groups, using One way Analysis of Variance, did not demonstrate a significant difference (p value: 0.9483).

Following the administration of isoproterenol, there was one death each in the disease control group (Group I) and the low dose metformin group (Group III). The number of animals that were available for further evaluation, on Day 10 were 7 in Group I and III and 8 in Group II and IV.

Biochemical evaluation results

Troponin T test

The number of animals that showed an elevation in the serum cardiac Troponin T levels (levels ≥ 0.08 ng/ml), as assessed using Roche Trop T Sensitive test kit, is mentioned in Table I.

Carrying out the contingency analysis of the findings using the 'Fisher Freeman Halton test', the p value was found to be 0.66, considered to be statistically non-significant.

Serum lactate dehydrogenase

The values of serum Lactate Dehydrogenase enzyme amongst the various study groups, expressed as Mean±Standard deviation, are as shown in Fig. 1.

TABLE I : Trop T test results: values expressed as absolute numbers.

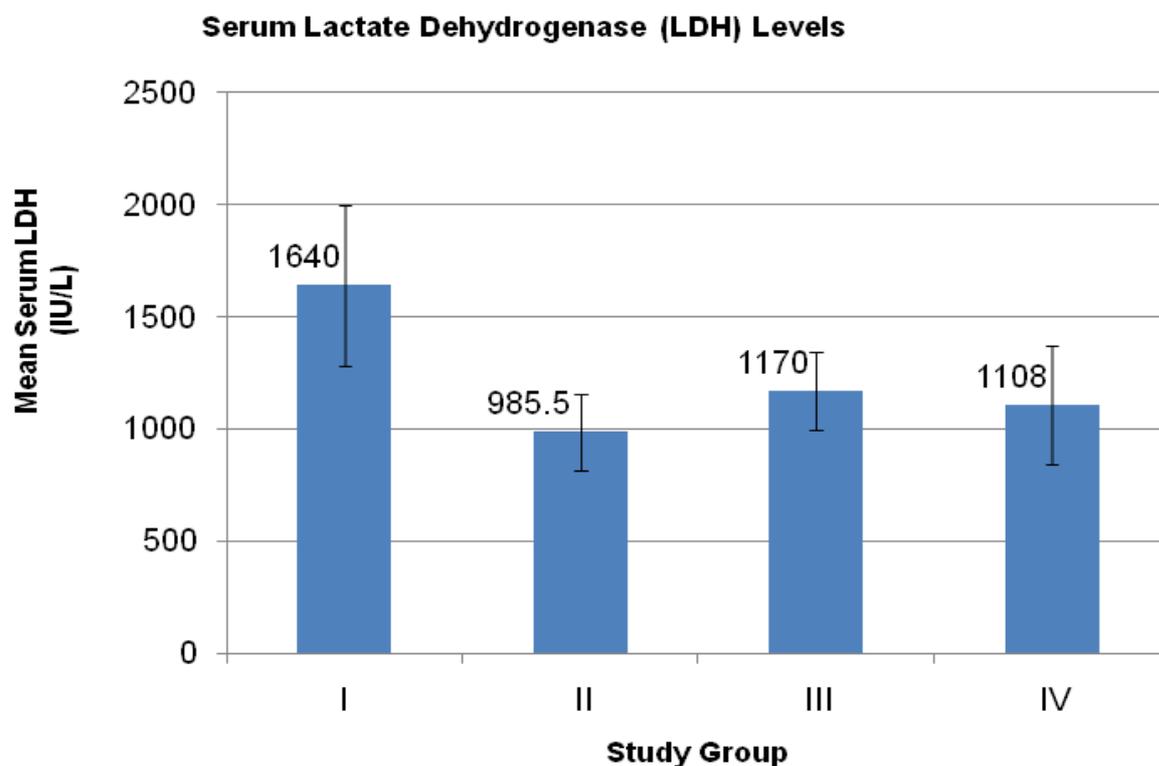| Group	Trop T positive	Trop T negative	Total
I	6	1	7
II	4	4	8
III	5	2	7
IV	5	3	8

Fig. 1 : Serum Lactate Dehydrogenase levels.
(Bars represent mean values; error-bars represent standard deviation)

Applying One Way Analysis of Variance, the difference between the mean enzyme levels for study groups, was found to be statistically significant, with p value of 0.0002.

Applying Tukey's post test, the findings are as summarized in Table II.

The mean serum lactate dehydrogenase level, as compared to the disease control group (Group I), was lower in the carvedilol (Group II), metformin low dose (Group III) and metformin high dose (Group IV) study groups, and this difference was significant on statistical analysis.

The mean enzyme level in Group II was lower as compared to Group III and Group IV; however, the differences among these groups were not statistically significant.

The correlation analysis between the serum lactate dehydrogenase levels and Trop T test results was carried out, using the Biserial correlation coefficient

TABLE II : Serum Lactate Dehydrogenase levels : Tukey's post test.

Tukey's Post Test	Mean difference (IU/L)	p<0.05 (Significance)
Group I vs Group II	654.5	Yes
Group I vs Group III	470.1	Yes
Group I vs Group IV	532.5	Yes
Group II vs Group III	−184.4	No
Group II vs Group IV	−122.0	No
Group III vs Group IV	62.36	No

(r_b). The overall mean for the serum lactate dehydrogenase levels was 1213.77 with a standard deviation (S_x) of 344.52 (IU/L).

The Biserial correlation coefficient r_b was found to be 0.8754. The two tailed p value was 0.00007, considered to be statistically significant.

Pathological evaluation

Heart Coefficient (Heart : Body weight ratio)

The observations are summarized as described in Fig. 2.
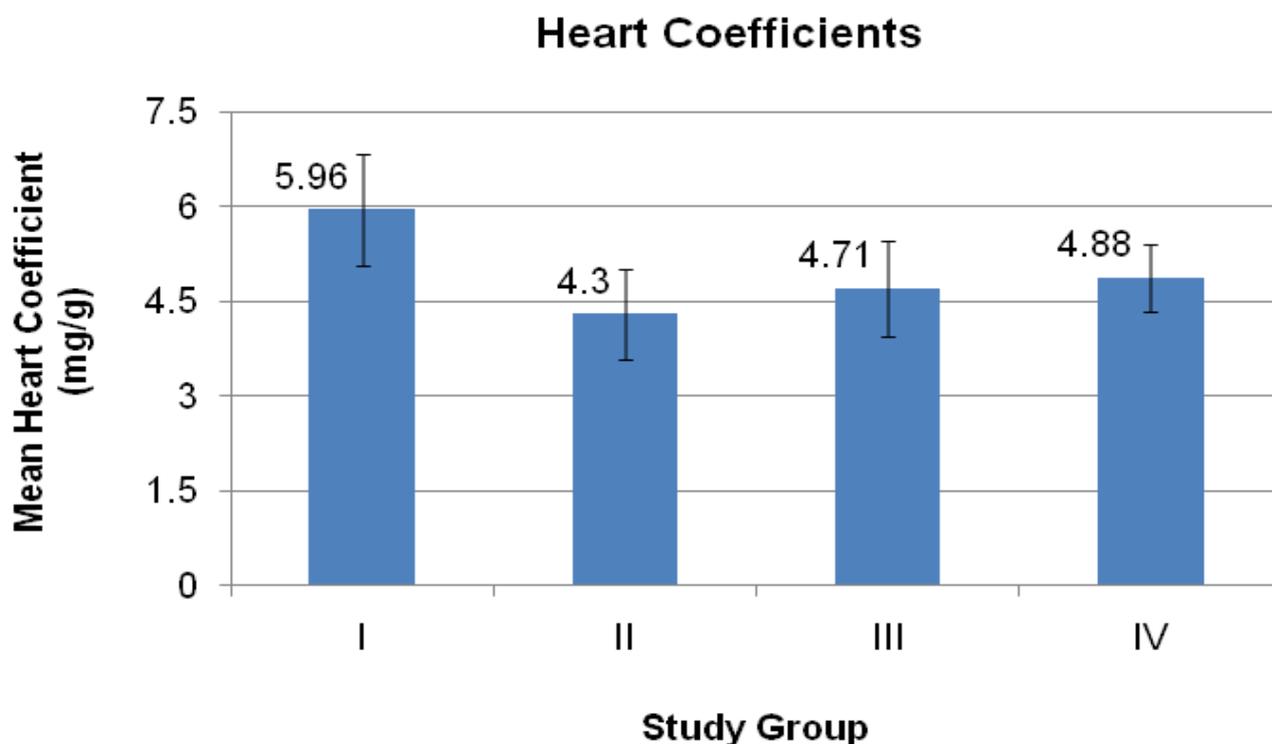

Heart Coefficients

Fig. 2 : Heart Coefficients of the Study Groups.
(Bars represent mean values; error-bars represent standard deviation)

Comparing the observations using 'One Way Analysis of Variance', the results demonstrated a statistically significant difference in the heart-coefficients between the study groups, with a p value of 0.0013. Applying Tukey's post test, the findings were as summarized in Table III.

Histopathology

Observations of histopathological grading are summarized in Fig. 3.

Applying the Kruskal-Wallis test for the difference in the medians between the study groups, the p value was found to be 0.0026, considered to be statistically significant.

TABLE III : Heart Coefficients: Tukey'spost test.

Tukey's Post Test	Mean difference (mg/g)	p<0.05 (Significance)
Group I vs Group II	1.661	Yes
Group I vs Group III	1.248	Yes
Group I vs Group IV	1.075	Yes
Group II vs Group III	−0.4126	No
Group II vs Group IV	−0.5858	No
Group III vs Group IV	−0.1733	No

Applying the post test, 'Dunn's Multiple Comparison Test', the differences between the median values of Group I and Group II, Group I and Group III as well as Group I and Group IV, were observed to be statistically significant. The median reading of Group II was lower than the median reading of Group III, as well as the median reading of Group IV; however, these differences were not found to be statistically significant. The results have been summarized in Table IV.

TABLE IV : Histopathological Grading: Dunn's multiple comparison test.

Dunn's multiple comparison test	Difference in rank sum	p<0.05 (Significance)
Group I vs Group II	14.75	Yes
Group I vs Group III	12.29	Yes
Group I vs Group IV	12.00	Yes
Group II vs Group III	−2.464	No
Group II vs Group IV	−2.750	No
Group III vs Group IV	−0.2857	No

Discussion

Metformin is clinically used as an anti-hyperglycemic drug, in patients of Type II Diabetes Mellitus. The
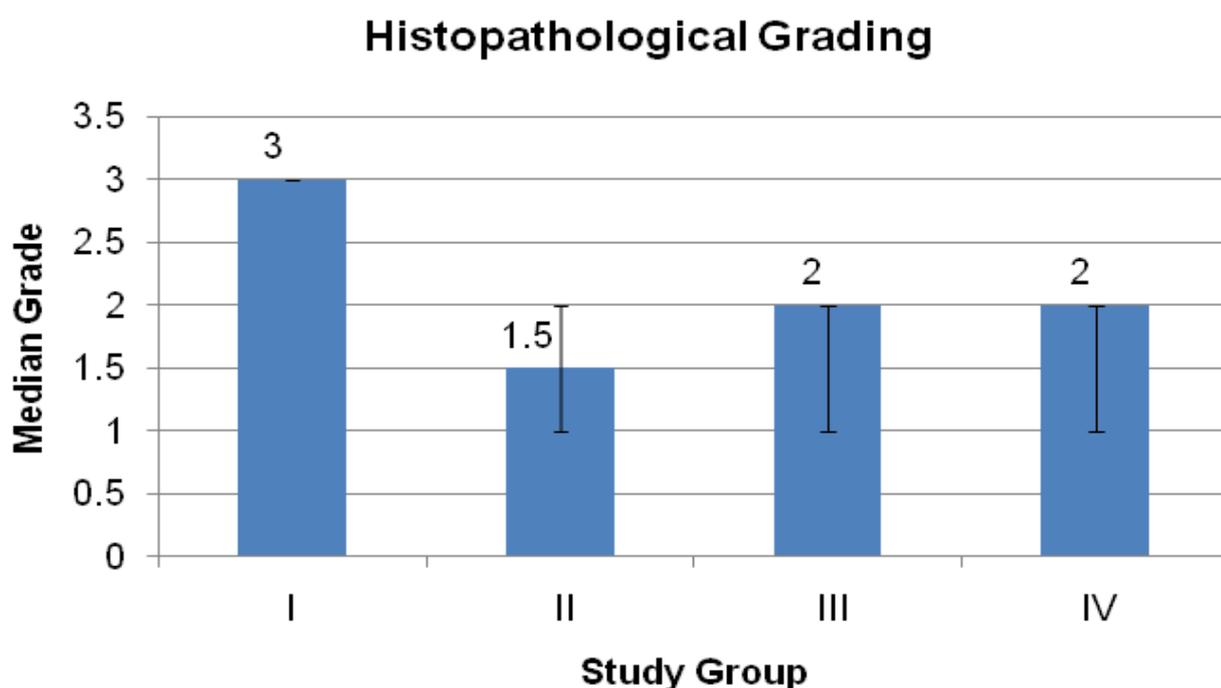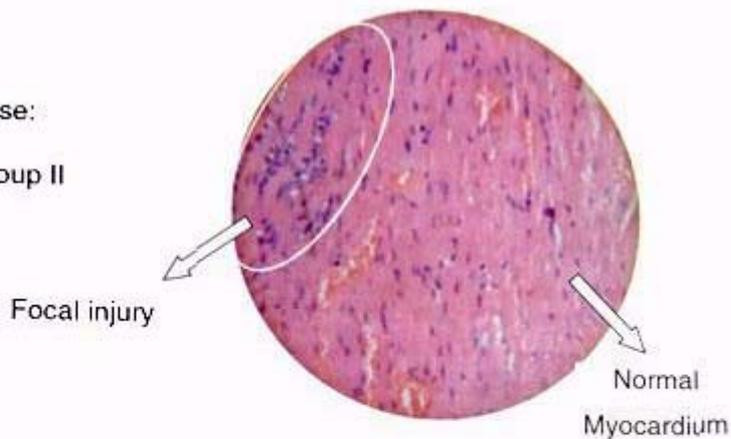

Histopathological Grading

Fig. 3 : Histopathological grading of myocardial injury for the study groups.
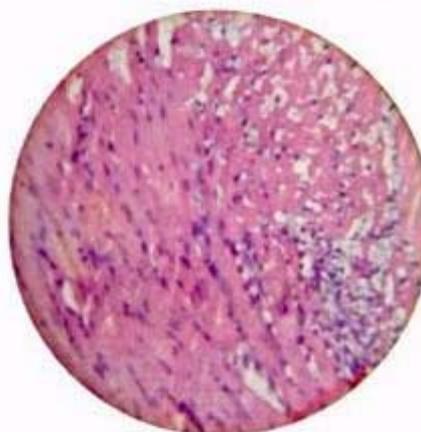(Bars represent mean values; error-bars represent standard deviation)

Grade I injury

Focal interstitial response:

Observed in a rat of Group II

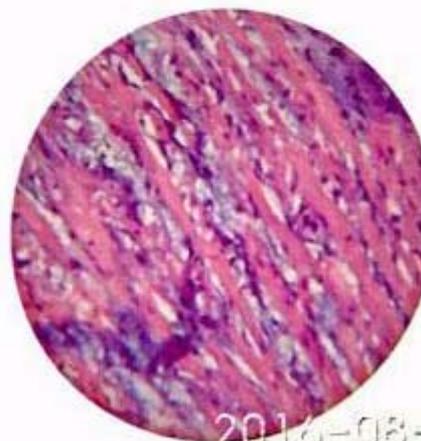Focal injury

Normal
Myocardium

Grade II injury

Focal lesions, with mottled staining and fragmentation of muscle fibres:

Observed in a rat of Group III

Grade III injury

Confluent retrogressive lesions with hyaline necrosis and fragmentation of muscle fibres and sequestrating mucoid edema:

Observed in a rat of Group I

HISTOPATHOLOGICAL OBSERVATIONS

hypothesis, of a cardioprotective effect of metformin therapy, was first generated during the analysis of the 'United Kingdom Prospective Diabetes Study - 34' (U.K.P.D.S. 34) outcomes (3). Since then, a few researchers have tried to test this hypothesis in various clinical (16, 17) and experimental studies (18-20). There have been some positive findings, as well as opinions against some of these findings, and the hypothesis remains to be conclusively proven (3, 16, 21, 22, 17-20).

The purpose of this study was to evaluate the hypothesized cardioprotective effect of metformin therapy, in a controlled experimental setting. The findings of our study are in support of the hypothesis, as in our study, prior metformin therapy for seven days, was associated with protection against a subsequent myocardial insult.

For the evaluation, we selected an animal study design, as an animal study permits more stringent control of the experimental conditions. This minimizes the chances of inter-individual variability, which may be observed in a clinical situation. Experiments done in animals, being more physiological in nature, also provide an advantage over the in-vitro studies (23). The experimental model of isoproterenol induced myocardial injury in rats was selected, based on its simplicity, feasibility and well established evidence in the evaluation of cardioprotection (24-26). For the study, carvedilol was selected as a known cardioprotective drug, due to its pleiotropic beneficial effects relevant to our model, including antioxidant mechanisms, β adrenergic receptor blockade, calcium channel blockade, vasodilation and anti-arrhythmic effects (6, 27, 28-30).

Excess body weight is a known risk-factor for cardiovascular morbidity (1), and metformin therapy is known to prevent an increase in the body weight (2, 8). Hence this effect, if present, could have influenced the findings of the study, in favour of the metformin treated groups. The weight gain in the different study groups was analyzed, during the course of administration of the respective compounds. The mean change in the body weight during this period was not found to be significantly different between the various study groups, on statistical analysis.

In our study, the evaluation of myocardial injury was carried out on the basis of biochemical parameters and pathological parameters.

The statistical analysis of the histopathological grading implies a protection to the myocardium, in terms of a reduction in the median grade, observed with both the low dose and high dose of metformin

used in our study. The extent of protection, observed with both these doses of metformin, was statistically similar to that observed with carvedilol.

The analysis of the Heart Coefficient values, indicates a protection to the myocardium, offered by both the low dose and the high dose of metformin used in our study. The extent of protection, observed with both these doses of metformin, was similar to that observed with carvedilol, on statistical analysis.

From the Trop T test, it could be interpreted that neither metformin (in either low dose or high dose), nor the standard drug carvedilol, were able to reduce the incidence of myocardial injury, as compared to the disease control group, on statistical analysis.

In regards to quantitative estimation of the serum lactate dehydrogenase levels, metformin, in both the doses used in our study, was found to have a protective effect on the myocardium. The decrease in the serum enzyme levels reflects a reduction in the severity of myocardial injury. The protection offered by either dose of metformin in our study, was statistically similar to that offered by carvedilol.

Recent studies done in murine models of myocardial infarction conclude that the administration of metformin potently limits infarct size. Activation of adenosine monophosphate-activated protein kinase, increased formation of adenosine, and the prevention of opening of the mitochondrial permeability transition pore at reperfusion all contribute to this cardioprotective effect. In addition, metformin therapy attenuates post infarction cardiac remodeling. Cardioprotection by Metformin is largely mediated by activation of AMP-activated protein kinase (AMPK), a key molecule orchestrating many biochemical processes such as glucose uptake, glycolysis, oxidation of free fatty acids and mitochondrial biogenesis. These processes significantly contribute to raise ATP levels and restore myocardial contractile efficiency. AMPK also activates endothelial nitric oxide synthase and promotes autophagy, thus preventing inflammation and cellular death. A reduced collagen expression also contributes to this effect (31, 32).

A study conducted by Haleh Vaez et al investigated the effect of metformin on myocardial dysfunction and TLR4 activity in LPS-induced sepsis by studying the concentration of TNF-α, content of MYD88, the phosphorylation of AMPK, and the rate of TLR4 expression. The results suggested that metformin exhibits cardioprotective effects in sepsis by suppression of TLR4 activity, at least in part through pathways involving AMPK activation (34). Metformin could also protect the aged, diabetic heart against ischemia-reperfusion injury (IRI) by up-regulating PGC-1α, a key controller of energy metabolism in skeletal muscle and thus improving the impaired functionality of diabetic mitochondria (33).

Although our study was not designed to elucidate the molecular mechanism of any possible cardioprotective action of metformin, the findings of our study are suggestive of its beneficial effect on the cardiovascular system. The cardioprotective effect of metformin, observed in our study, is unlikely due to its anti-hyperglycemic action. This can be judged by the fact that the study was carried out in the experimental subjects (rats) with normal baseline blood glucose levels, and in stringent experimental conditions. Also, the observed cardioprotective effect of metformin is unlikely due to its effect on body weight control, as the mean changes in the body weights during the period of administration of the compounds, were not found to be significantly different between the study groups, on statistical analysis.

Limitations of the study

Mortality may be expected in our study model of myocardial injury. This may occur even with cardioprotection due to the study drug, as the protection may not completely prevent the myocardial damage or the development of arrhythmias. The effect of a study drug, on the incidence of mortality, needs to be evaluated in a different manner. This may be possible by studying the effect of the drug on the median lethal dose (LD - 50) of isoproterenol. Our study was not designed for this purpose. Hence, a further evaluation is required to identify the effect of prior metformin therapy, on the incidence of cardiac-related mortality, in experimental animals.

The incidence of myocardial injury was not found to be significantly reduced on statistical analysis. This may be attributed to the small sample size used in our study. Also, as described by Rona et al, the compounds were administered for a short term (7 days) prior to the induction of myocardial injury (5). The outcome may differ, if the compounds were administered for a longer duration. This hypothesis may be tested in further studies.

Conclusion

From the observations of the study, it may be concluded that metformin administration for a short term (7 days), prior to a subsequent isoproterenol induced myocardial injury, demonstrates a cardioprotective effect, in terms of reduction in the degree of myocardial injury, in normoglycemic rats.

This observation may have clinical relevance, as it may be developed as a new therapeutic approach for cardiovascular risk prevention, with further establishment of the evidence in a clinical setting.

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