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Possible involvement of caveolin in attenuation of cardioprotective effect of ischemic preconditioning in diabetic rat heart

Preeti Ajmani, Harlokesh N Yadav[*], Manjeet Singh and Pyare L Sharma

Abstract

Background: Nitric oxide (NO) has been noted to produce ischemic preconditioning (IPC)-mediated cardioprotection. Caveolin is a negative regulator of NO, which inhibits endothelial nitric oxide synthase (eNOS) by making caveolin-eNOS complex. The expression of caveolin is increased during diabetes mellitus (DM). The present study was designed to investigate the involvement of caveolin in attenuation of the cardioprotective effect of IPC during DM in rat.

Methods: Experimental DM was induced by single dose of streptozotocin (50 mg/Kg, *i.p,*) and animals were used for experiments four weeks later. Isolated heart was mounted on Langendorff's apparatus, and was subjected to 30 min of global ischemia and 120 min of reperfusion. IPC was given by four cycles of 5 min of ischemia and 5 min of reperfusion with Kreb's-Henseleit solution (K-H). Extent of injury was measured in terms of infarct size by triphenyltetrazolium chloride (TTC) staining, and release of lactate dehydrogenase (LDH) and creatin kinase-MB (CK-MB) in coronary effluent. The cardiac release of NO was noted by measuring the level of nitrite in coronary effluent.

Results: IPC- induced cardioprotection and release of NO was significantly decreased in diabetic rat heart. Pre-treatment of diabetic rat with daidzein (DDZ) a caveolin inhibitor (0.2 mg/Kg/s.c), for one week, significantly increased the release of NO and restored the attenuated cardioprotective effect of IPC. Also perfusion of sodium nitrite (10 μM/L), a precursor of NO, significantly restored the lost effect of IPC, similar to daidzein in diabetic rat. Administration of 5-hydroxy deaconate (5-HD), a mito K_{ATP} channel blocker, significantly abolished the observed IPC-induced cardioprotection in normal rat or daidzein and sodium nitrite perfused diabetic rat heart alone or in combination.

Conclusions: Thus, it is suggested that attenuation of the cardioprotection in diabetic heart may be due to decrease the IPC mediated release of NO in the diabetic myocardium, which may be due to up -regulation of caveolin and subsequently decreased activity of eNOS.

Background

Ischemic heart disease is a leading cause of morbidity and mortality worldwide [1]. Reperfusion of an ischemic myocardium is a requisite, for the restoration of the normal functioning of the myocardium [2]. However, abrupt reperfusion of an ischemic myocardium is not without hazard; it produces further damage of myocardium, described as ischemia-reperfusion (I/R) injury

[3,4]. Moreover, it has been documented that "controlled reperfusion" avoids further injury, both in myocardium and in peripheral tissues [5-8]. Brief episodes of ischemia followed by reperfusion of myocardium, increase the resistance against sustained ischemia of longer duration; this phenomenon is termed as ischemic preconditioning (IPC) [9]. IPC produces cardioprotection by PI-3K/Akt [10,11], phosphorylation of eNOS and by generation of nitric oxide (NO) and by opening of mito K_{ATP} channel [12,13]. However, the cardioprotective effect of IPC is attenuated in conditions such as heart failure [14,15] aging [16,17] hypertension ([18,19]

* Correspondence: hnyadav@gmail.com
Department of Pharmacology, Indo-Soviet College of Pharmacy, Moga 142-001, Punjab, India

obesity [20] hyperlipidemia [21-23]and diabetes mellitus [24-26]. Diabetes mellitus is a one of the major risk factor for ischemic heart disease.

Caveolin is the caveolar membrane protein, invaginated on the plasma membrane that serves as signalling platform for many of the G-protein coupled receptors (GPCR) [27-29]. IPC exerts cardioprotection by impairing the death signalling components p38MAPKα and JNK [30], by increase its association with caveolin. It has been well documented that caveolin is a negative regulator of eNOS, it interacts and inhibits the activity of eNOS by making caveolin-eNOS complex [31,32]. IPC increases the activity of eNOS by disrupting the complex of caveolin and eNOS in rat heart [32]. Moreover, it has been documented that NO produces cardioprotection by opening of K_{ATP} channel during IPC, and caveolin facilitates the interaction of NO with K_{ATP} channel by forming a suitable signaling platform [33]. Caveolin maintains eNOS in inactivated state and thereby limits NO production [34,35] and on agonist stimulation leads to activation of eNOS through increased disruption of caveolin/eNOS complex [31].

In diabetic rat heart, expression of caveolin increases [36-38] which enhances the binding of eNOS to caveolin and decreases the release of NO [31]. Therefore, the present study was undertaken to elucidate whether or not the diminished eNOS/NO signaling in diabetic myocardium is responsible for loss of cardioprotective effect of IPC.

Methods
The experimental protocol used in the present study was approved by Institutional Animal Ethics Committee.

Drugs and chemicals
Daidzein (0.2 mg/Kg/s.c) (Sigma Aldrich [P] Ltd., Bangalore, India) was dissolved in dimethyl sulfoxide (DMSO) and then injected to the animals for 7 days, 3 weeks after the administration of streptozotocin. Sodium nitrite (10 μM/L) (Rankem, Fine Chemicals Ltd., New Delhi, India) and 5-Hydroxy Decanoate (100 μM/L) (Sigma Aldrich [P] Ltd., Bangalore, India) were added in minimum quantity of distilled water and added to Kreb's Henseleit solution. All other reagents used in this study were of analytical grade and always freshly prepared before use.

Induction of experimental diabetes
Total 12 groups have been used in present study each group consist of 6-10 Wistar rats (180-250) of either sex. Experimental diabetes was induced by single dose administration of streptozotocin (50 mg/kg, *i.p*) [39]. There was 10% of mortality within 1st week and 20% mortality was noted up to harvesting of heart. Serum

glucose was estimated spectrophotometrically at 505 nm by glucose oxidase/pyruvate oxidase (GOD-POD) method [40,41] using an enzymatic kit (Kamineni Life Sciences Pvt. Ltd. Hyderabad, India). Serum glucose level > 200 mg/dl were considered to be hyperglycaemic.

Isolated rat heart preparation
Rats were administered heparin (500 IU/L, i.p) 20 min. prior to sacrificing the animal by cervical dislocation. Heart was rapidly excised and immediately mounted on Langendorff's apparatus [42]. Isolated heart was retrogradely perfused at constant pressure of 80 mmHg with Kreb's-Henseleit (KH) buffer (NaCl 118 mM; KCl 4.7 mM; CaCl2 2.5 mM; MgSO4.7H20 1.2 mM; KH2PO4 1.2 mM; C6H12O6 11 mM), pH 7.4, maintained at 37°C bubbled with 95% O2 and 5%CO2. Flow rate was maintained at 7-9 ml/min. using Hoffman's screw. The heart was enclosed in double wall jacket, the temperature of which was maintained by circulating water heated at 37° C. Ischemic preconditioning was produced by closing the inflow of K-H solution for 5 min followed by 5 min of reperfusion. Four such episodes were employed. Global ischemia was produced for 30 min. followed by 120 min. of reperfusion. Coronary effluent was collected before ischemia, immediately, 5 min. and 30 min. after reperfusion for estimation of Lactate Dehydrogenase (LDH) and Creatine Kinase (CK-MB) [43].

Assessment of myocardial injury
The assessment of myocardial infarct size was done by using triphenyltetrazolium chloride (TTC) staining method, while LDH and CK-MB were estimated by using commercially available kits (LDH Siemens Medical Solution Diagnostics Ltd., Ajwa Road, Baroda, India, CK-MB Nicholas Piramal India Ltd., Mumbai). Values of LDH and CK-MB were expressed in international units per litre (IU/L).

Assessment of myocardial infarct size
The heart was removed from the Langendorff's apparatus. Both the atria and root of aorta were excised and ventricles were kept overnight at -4°C temperature. Frozen ventricles were sliced into uniform sections of about 1-2 mm thickness. The slices were incubated in 1% w/v triphenyltetrazolium chloride stain (TTC stain) at 37°C in 0.2 M Tris-chloride buffer for 30 min. The normal myocardium was stained brick red while the infarcted portion remained unstained. Infarct size was measured by the volume method [44]
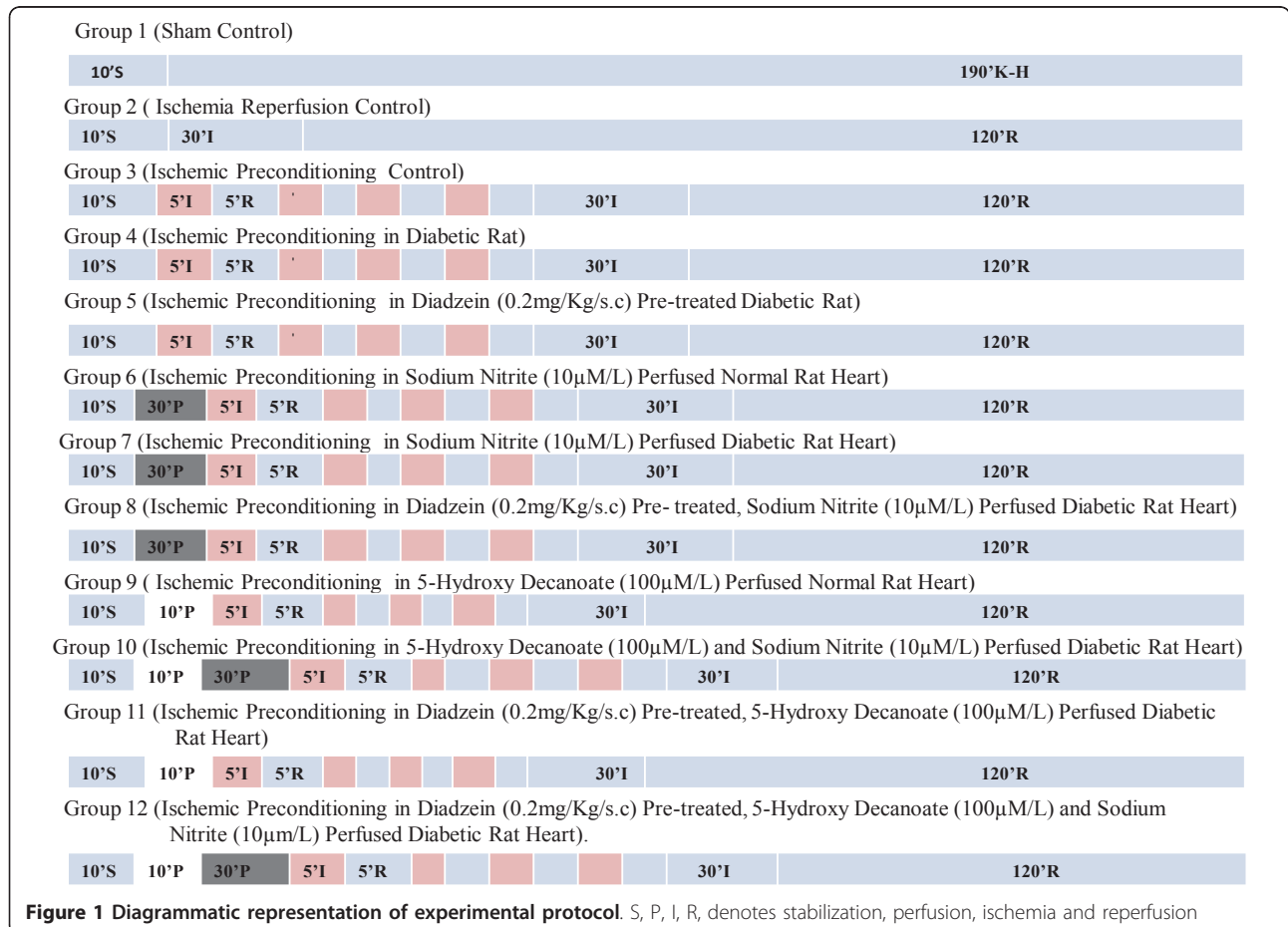
Nitrite estimation
Nitrite is a stable nitrogen intermediate formed from the spontaneous degradation of NO. Unlike NO, nitrite can be measured easily and nitrite concentrations can be

used to infer levels of NO production [45-47]. Nitrite release in coronary effluent was measured [48]. Greiss reagent 0.5 ml (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride in water) was added to 0.5 ml of coronary effluent. The optical density at 550 nm was measured using spectrophotometer (UV-1700 Spectrophotometer, Shimadzu, Japan). Nitrite concentration was calculated by comparison with spectrophotometer reading of standard solution of sodium nitrite prepared in K-H buffer [48]

Experimental protocol

A diagrammatic representation of experimental protocol is shown in Figure 1. In all groups, isolated rat heart was perfused with K-H solution and allowed for 10 min of stabilization. Group 1 (Sham Control; n = 6): Isolated rat heart was perfused continuously for 200 min without subjecting them to global ischemia and reperfusion. Group 2 (Ischemia-Reperfusion Control; n = 6): After 10 min of stabilization, isolated rat heart preparation was subjected to 30 min. global ischemia followed by 120 min. of reperfusion. Group 3 (Ischemic Preconditioning

Control; n = 6): After 10 min of stabilization, heart was subjected to four cycles of ischemic preconditioning, each cycle comprised of 5 min. global ischemia followed by 5 min. reperfusion further followed by 30 min. global ischemia and 120 min. of reperfusion. Group 4 (Ischemic Preconditioning in Diabetic Rats; n = 6): Isolated heart preparation from diabetic rat subjected to four cycles of ischemic preconditioning as described earlier in group 3. Group 5 (Ischemic Preconditioning in Daidzein (0.2 mg/Kg/s.c/day) Pre-treated Diabetic Rat; n = 6): Isolated rat heart preparation from daidzein (0.2 mg/Kg/s.c/day) pre-treated diabetic rat was subjected to four cycles of ischemic preconditioning as described earlier in group 3. Group 6 (Ischemic preconditioning in Sodium Nitrite (10 µM/L) perfused Normal Rat Heart; n = 6): After 10 min of stabilization, heart was perfused with K-H buffer containing sodium nitrite (10 µM/L) for 30 min. and then subjected to four cycles of ischemic preconditioning as described earlier in group 3. Group 7 (Ischemic preconditioning in Sodium Nitrite (10 µM/L) perfused Diabetic Rat Heart; n = 6): Isolated heart preparation obtained from diabetic rat was perfused with sodium nitrite (10 µM/L) for 30 min. followed by IPC as described in group

Figure 1 Diagrammatic representation of experimental protocol. S, P, I, R, denotes stabilization, perfusion, ischemia and reperfusion

3. Group 8 (Ischemic Preconditioning in Daidzein (0.2 mg/Kg/s.c/day) Pre-treated, Sodium Nitrite (10 μM/L) Perfused Diabetic Rat Heart; n = 6): After 10 min of stabilization, Isolated rat heart preparation from daidzein (0.2 mg/Kg/s.c/day) pre-treated diabetic rat was perfused with sodium nitrite (10 μM/L) for 30 min. followed by as described in group 3. Group 9 (Ischemic Preconditioning in 5-Hydroxy Decanoate (100 μM/L) Perfused Normal Rat Heart; n = 6): After 10 min of stabilization, isolated heart was perfused with K-H buffer containing 5-hydroxy decanoate (100 μM/L) for 10 min. and then subjected to IPC as described earlier in group 3. Group 10 (Ischemic Preconditioning in Daidzein (0.2 mg/Kg/s.c/day) Pre-treated, 5-Hydroxy Decanoate (100 μM/L) Perfused Diabetic Rat Heart; n = 6): After 10 min of stabilization, isolated rat heart preparation from daidzein (0.2 mg/Kg/s.c/day) pre-treated diabetic rat perfused with 5-hydroxy decanoate (100 μM/L) containing K-H buffer for 10 min. followed by as described in group 3. Group 11 (Ischemic Preconditioning in 5-Hydroxy Decanoate (100 μM/L) and Sodium Nitrite (10 μM/L) Perfused Diabetic Rat Heart; n = 6): After 10 min of stabilization, isolated heart preparation from diabetic rat was perfused with K-H buffer containing 5-hydroxy decanoate (100 μM/L) for 10 min. which is further followed by perfusion with sodium nitrite (10 μM/L) for 30 min. followed by as described earlier in group 3. Group 12 (Ischemic Preconditioning in Daidzein (0.2 mg/Kg/s.c/day) Pre-treated, 5-Hydroxy Decanoate (100 μM/L) and Sodium Nitrite (10 μM/L) Perfused Diabetic Rat Heart; n = 6): After 10 min of stabilization, isolated rat heart preparation from daidzein (0.2 mg/Kg/s.c/day) treated diabetic rat was followedby group 11.

Statistical analysis

All values were expressed as mean ± standard deviation (S.D). Statistical analysis was performed using Sigmastat Software. Glucose value was compared by Student's paired t-test. The values of infarct size, LDH, CK-MB and nitrite level were statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test as a post hoc test. Value of $P < 0.05$ was considered to be statistically significant.

Results

Effect of streptozotocin on serum glucose

The administration of single dose of streptozotocin (50 mg/Kg, *i.p*) significantly increased blood glucose as compared to basal value (Figure 2).

Effect of ischemic preconditioning and pharmacological interventions on myocardial infarct size

Global ischemia for 30 min followed by 120 min of reperfusion significantly increased the myocardial infarct size, as compared to sham control. Four episodes of IPC significantly decreased I/R induced increase in myocardial infarct size in normal rat heart. However, ischemic preconditioning failed to decrease the myocardial infarct size in diabetic rat heart. Moreover, IPC induced decrease of infarct size was significantly restored in DDZ pre-treated and in sodium nitrite perfused diabetic rat heart. However, perfusion with 5-HD significantly attenuated the decrease of myocardial infarct size in normal, DDZ pre-treated and sodium nitrite perfused diabetic rat heart alone or in combination (Figure 3).

Effect of ischaemic preconditioning and pharmacological interventions on the release of Lactate dehydrogenase (LDH)
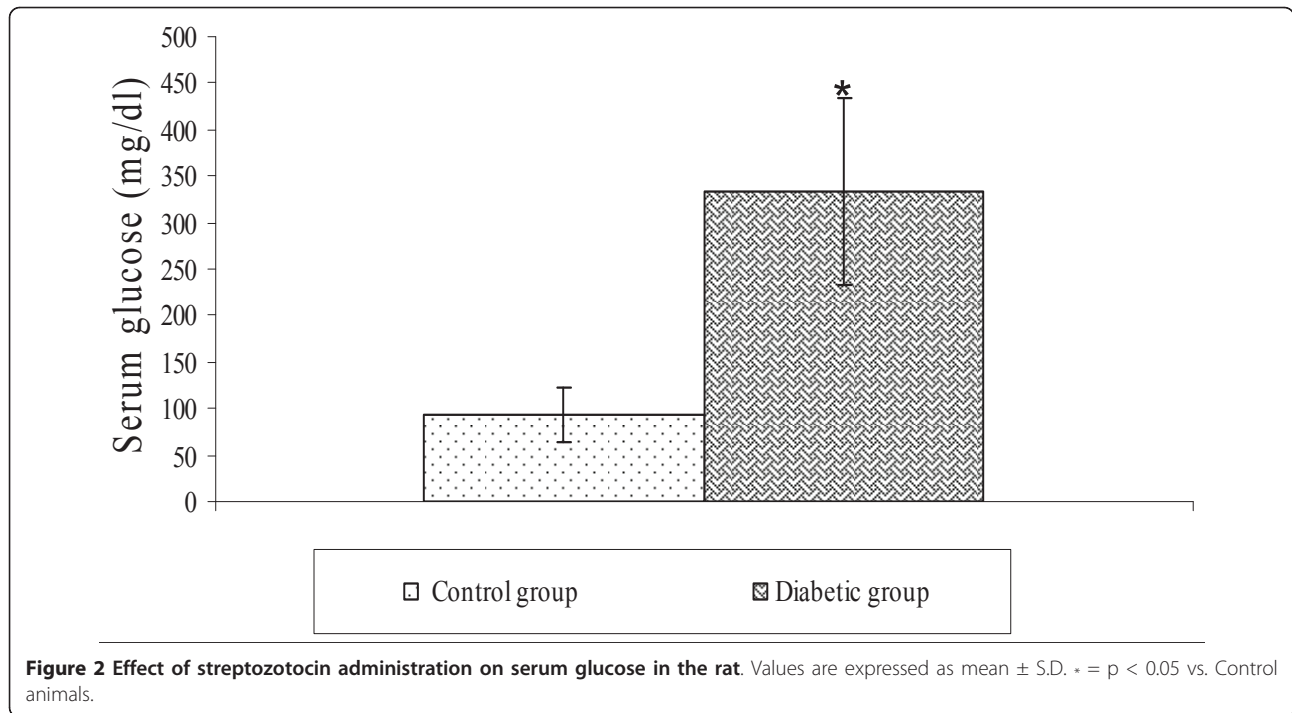
Global ischemia for 30 min followed by 120 min of reperfusion markedly increased the release of LDH as compared to sham control. Four episodes of IPC significantly reduced the I/R induced increase in the release of LDH in normal rat heart but not in the diabetic rat heart. Moreover, IPC induced decrease in the release of LDH was significantly restored in DDZ pre-treated, and in sodium nitrite perfused diabetic rat heart. However, perfusion with 5-HD significantly attenuated the decrease in the release of LDH in normal, DDZ pre-treated and sodium nitrite perfused diabetic rat heart alone or in combination (Figure 4).

Effect of ischemic preconditioning and pharmacological interventions on the release of CK-MB

Global ischemia for 30 min followed by 120 min of reperfusion markedly increased the release of CK-MB, as compared to sham control. Four episodes of IPC significantly reduced the I/R induced increase in the release of CK-MB in normal rat heart but not in the diabetic rat heart. Moreover, IPC induced decrease release of CK-MB was significantly restored in DDZ pre-treated and sodium nitrite perfused diabetic rat heart. However, perfusion with 5-HD significantly attenuated the decrease in the release of CK-MB in normal, DDZ pre-treated and sodium nitrite perfused diabetic rat heart alone or in combination (Figure 5).

Effect of ischemic preconditioning and treatment with daidzein on the release of nitrite
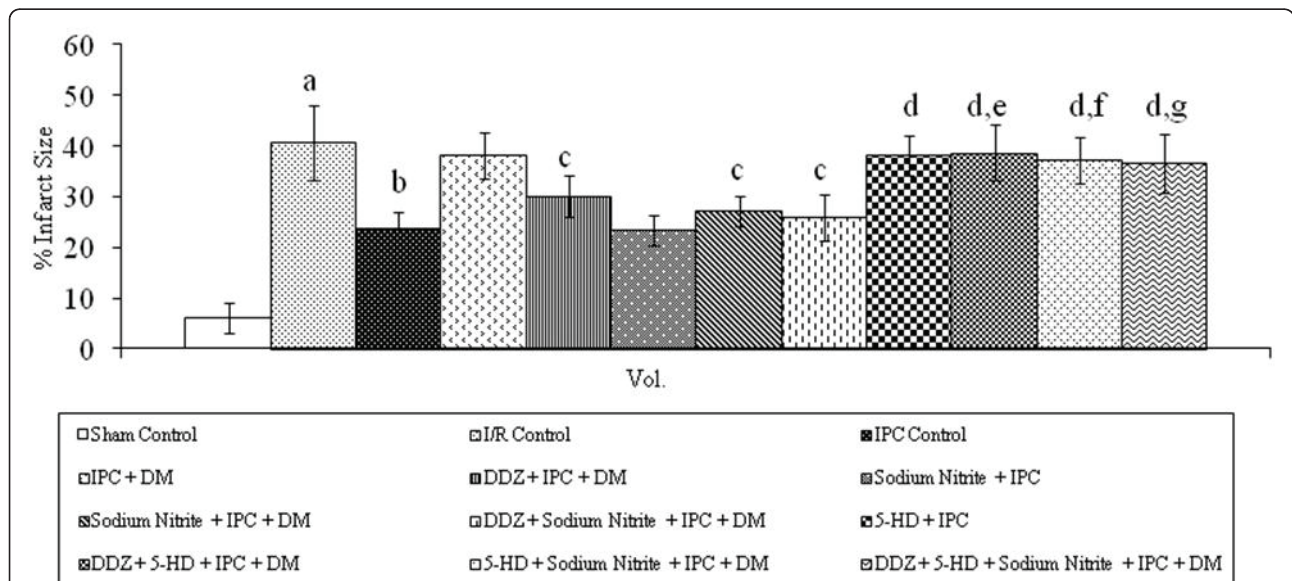
Four episodes of IPC significantly increased the release of nitrite into coronary effluent of normal animals, as compared to I/R group but not in isolated heart obtained from diabetic rat. Treatment with daidzein, a caveolin inhibitor (0.2 mg/Kg/s.c, one week), significantly increased, the release of nitrite in diabetic rat heart subjected to IPC (Figure 6).
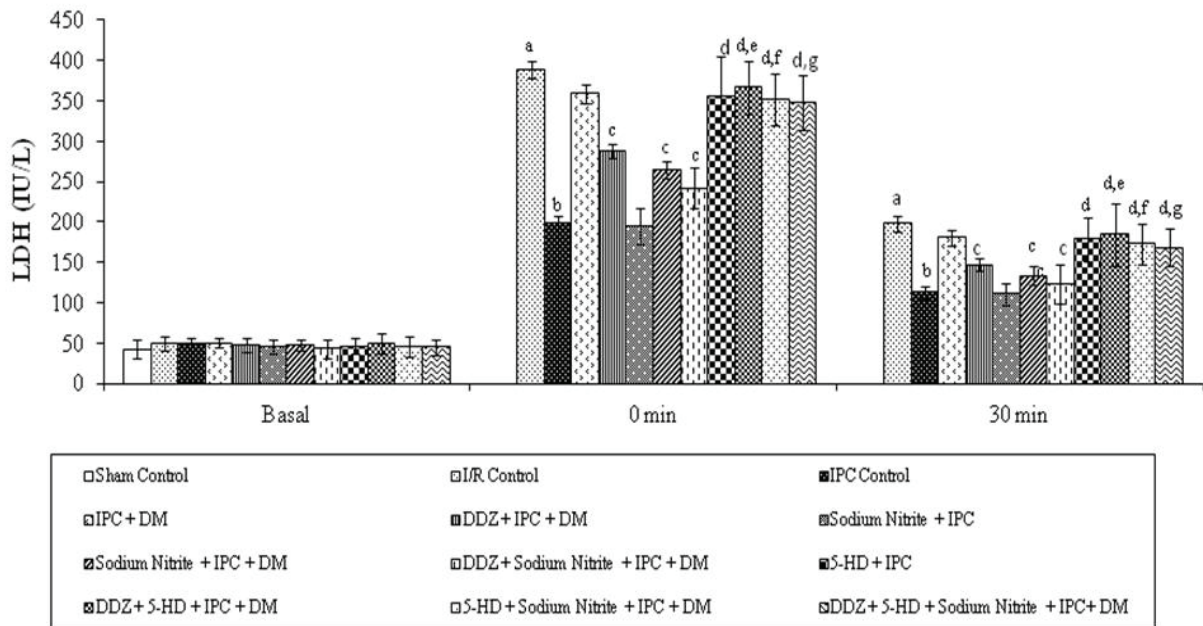
Figure 2 Effect of streptozotocin administration on serum glucose in the rat. Values are expressed as mean ± S.D. ∗ = p < 0.05 vs. Control animals.

Discussion

Four episodes of 5 min ischemia followed by reperfusion for 5 min, effectively preconditioned the normal rat heart as indicated by a significant decrease in infarct size and ischemia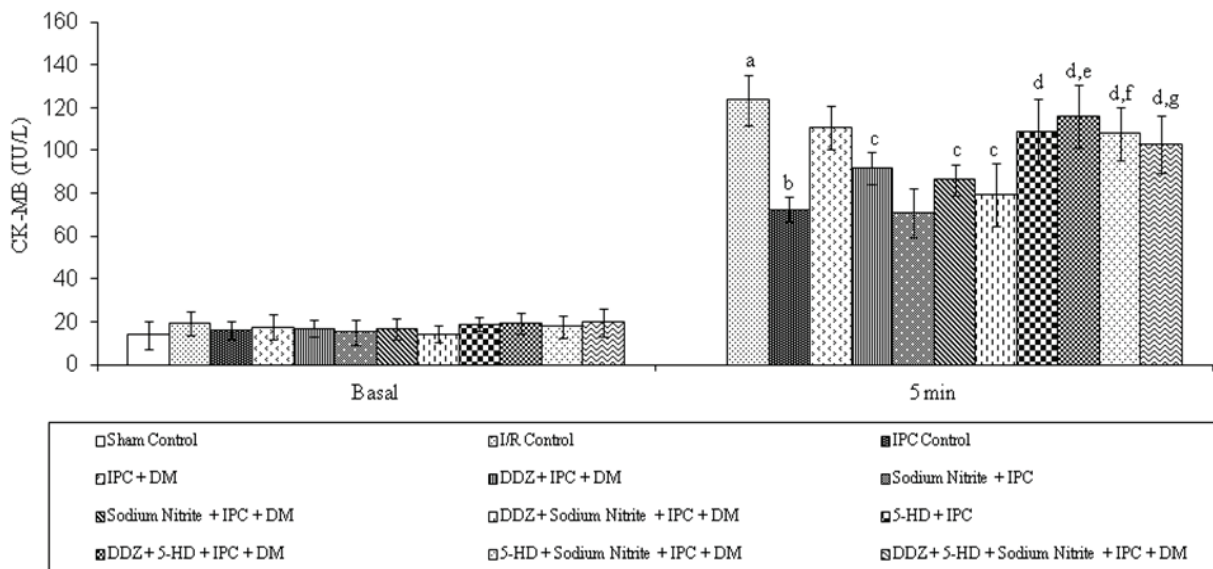-reperfusion induced release of LDH and CK-MB. This cardioprotective effect of ischemic preconditioning is in agreement with earlier studies [49-51]. However the cardioprotective effect of ischemic preconditioning was significantly attenuated in diabetic rat heart. Our result is supported by earlier published
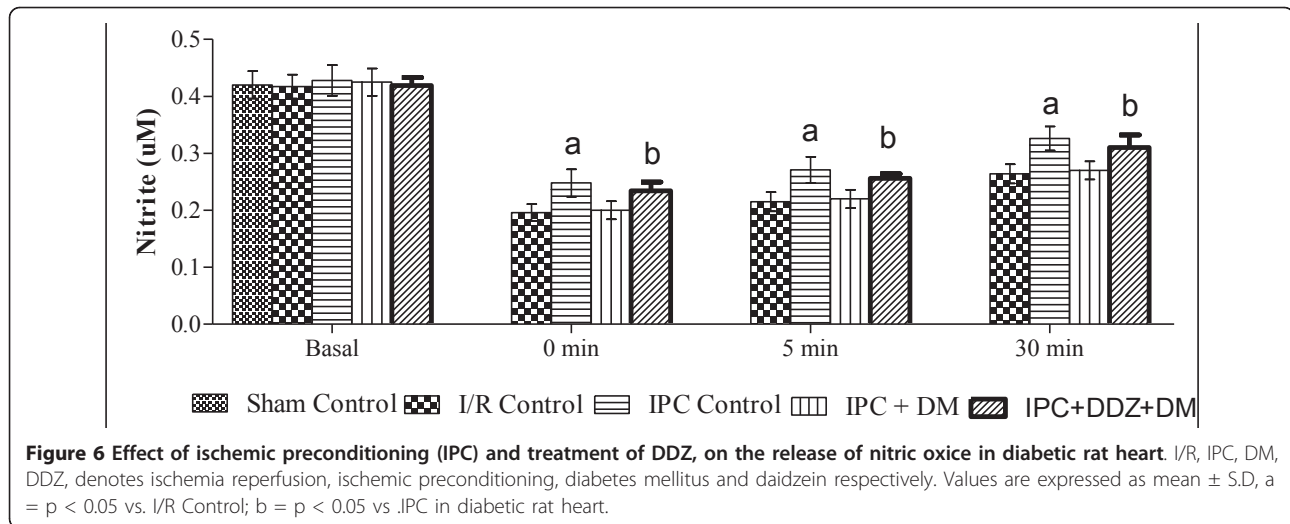

Figure 3 Effect of I-R on myocardial infarct size, effect of ischemic preconditioning (IPC) on myocardial infarct size in normal and diabetic rat heart, effect of DDZ pre-treatment, sodium nitrite perfusion on myocardial infarct size in diabetic rat heart and effect of 5-HD alone or in combination with DDZ and sodium nitrite on myocardial infarct size in diabetic rat heart. I/R, IPC, DM, DDZ, 5-HD denotes ischemia reperfusion, ischemic preconditioning, diabetes mellitus, daidzein and 5-hydroxy decanoate respectively. Values are expressed as mean ± S.D, a = p < 0.05 vs. sham control; b = p < 0.05 vs. I/R Control; c = p < 0.05 vs .IPC in diabetic rat heart; d = p < 0.05 vs. IPC in normal rat heart; e = P < 0.05 vs. IPC in DDZ pretreated diabetic rat heart; f = p < 0.05 vs. IPC in sodium nitrite perfused diabetic rat heart; g = p < 0.05 vs. IPC in DDZ pretreated, sodium nitrite perfused diabetic rat heart.

Figure 4 Effect of I-R on the release of LDH, effect of ischemic preconditioning (IPC) on the release of LDH in normal and diabetic rat heart, effect of DDZ pre-treatment, sodium nitrite perfusion on the release of LDH in diabetic rat heart and effect of 5-HD alone or in combination with DDZ and sodium nitrite on the release of LDH in diabetic rat heart. I/R, IPC, DM, DDZ, 5-HD denotes ischemia reperfusion, ischemic preconditioning, diabetes mellitus, daidzein and 5-hydroxy decanoate respectively. Values are expressed as mean ± S.D, a = p < 0.05 vs. sham control; b = p < 0.05 vs. I/R Control; c = p < 0.05 vs. IPC in diabetic rat heart; d = p < 0.05 vs. IPC in normal rat heart; e = P < 0.05 vs. IPC in DDZ pretreated diabetic rat heart; f = p < 0.05 vs. IPC in sodium nitrite perfused diabetic rat heart; g = p < 0.05 vs. IPC in DDZ pretreated, sodium nitrite perfused diabetic rat heart.

Figure 5 Effect of I-R on the release of CK-MB, effect of ischemic preconditioning (IPC) on the release of CK-MB in normal and diabetic rat heart, effect of DDZ pre-treatment, sodium nitrite perfusion on the release of CK-MB in diabetic rat heart and effect of 5-HD alone or in combination with DDZ and sodium nitrite on the release of CK-MB in diabetic rat heart. I/R, IPC, DM, DDZ, 5-HD denotes ischemia reperfusion, ischemic preconditioning, diabetes mellitus, daidzein and 5-hydroxy decanoate respectively. Values are expressed as mean ± S.D, a = p < 0.05 vs. sham control; b = p < 0.05 vs. I/R Control; c = p < 0.05 vs .IPC in diabetic rat heart; d = p < 0.05 vs. IPC in normal rat heart; e = P < 0.05 vs. IPC in DDZ pretreated diabetic rat heart; f = p < 0.05 vs. IPC in sodium nitrite perfused diabetic rat heart; g = p < 0.05 vs. IPC in DDZ pretreated, sodium nitrite perfused diabetic rat heart.

Figure 6 Effect of ischemic preconditioning (IPC) and treatment of DDZ, on the release of nitric oxice in diabetic rat heart. I/R, IPC, DM, DDZ, denotes ischemia reperfusion, ischemic preconditioning, diabetes mellitus and daidzein respectively. Values are expressed as mean ± S.D, a = $p < 0.05$ vs. I/R Control; b = $p < 0.05$ vs .IPC in diabetic rat heart.

studies [52-54,22,23]. It has been reported that perfusion of sodium nitrite produces cardioprotection in isolated heart from normal rat, subjected to global ischemia [55,56]. In our study, perfusion of sodium nitrite (a precursor of NO) [56] followed by IPC, significantly restored the attenuated effect of IPC in the diabetic myocardium (decreases in infarct size and in the release of LDH and CK-MB in coronary effluents). It is probable that the attenuated cardioprotective effect of IPC in diabetic rat may be due to decreased availability of NO. Thus, NO appears to be responsible for cardioprotective effect of ischemic preconditioning [13]. However, in our study, treatment with sodium nitrite did not enhance the cardioprotective effect of IPC in normal rat. This indicates that once IPC mediated increased generation of NO achieved the threshold for cardioprotection and the addition of sodium nitrite (NO donor) [56] was unable to further increase the myocardial protection by IPC, per se.

Opening of mitochondrial ATP sensitive potassium channels (mito K_{ATP} channels) protects the myocardium from ischemia-reperfusion induced injury [57]. Various mediators i.e. adenosine, bradykinin, angiotensin, prostaglandins and NO which are released by the stimuli of IPC produce cardioprotection through opening of mito K_{ATP} channel [58,59]. Further, administration of 5-HD, a mitoK_{ATP} channel blocker, attenuated the observed cardioprotective effect of IPC in normal rat heart and in the sodium nitrite perfused diabetic rat heart. It is suggested that the observed cardioprotective effect of IPC in normal rat and sodium nitrite perfused diabetic rat may be due to opening of mito K_{ATP} channel. Our results are in accordance with reports from other laboratories [60,61].

Caveolins are proteins that form the structure of caveolar membrane, act as a signaling platform (signalosomes) for molecules such as nitric oxide synthase (NOS) and Src-like kinases and many of the G-proteins coupled receptors (GPCR) [27-29]. Ischemic preconditioning can modulate the microenvironment of caveolin and promote the signalling involved in protection of myocardium against ischemia-reperfusion induced injury [62]. It has been reported that expression of caveolin is upregulated in diabetic myocardium [37,38]. Caveolin is known to be a negative regulator of NO, it maintains eNOS in inactivated state by making caveolin-eNOS complex [36] and on agonist stimulation leads to activation of eNOS and increased release of NO; by disrupting caveolin/eNOS complex [31]. Increased expression of caveolin may lead to the increased interaction with eNOS, decreasing it's phosphorylation and a consequent decrease in the generation of NO [63]. In our study, IPC-induced release of nitrite in diabetic rat was significantly decreased as compared to normal rat. Our finding is supported by other reports that the decreased release of NO in the diabetic rat heart, is due to decreased activity of eNOS by an upregulation of caveolin in the myocardium [36,38].

Treatment with daidzein, has been noted to inhibit the expression of a caveolin in the diabetic rat [64,65]. In the present study daidzein treatment for seven days, three weeks after the administration of STZ, followed by IPC; significantly restored the cardioprotective effect in diabetic rat heart and increased the release of NO, without affecting the serum glucose. In normal heart, IPC leads to increased expression of caveolae [66]. Each brief episode of coronary occlusion produces small bursts of reactive oxygen species (ROS), and leads to increased formation and release of NO, by cleaving the caveolin-eNOS complex. Furthermore, antioxidants have been demonstrated to abolish IPC-induced cardiac protection in normal heart (62,67,68). Why IPC-induced cardiac protection is lost in diabetic heart is not known?.

However, the restoration of IPC-induced cardiac protection by daidzein pre-treatment indicates that some defect in caveolin-eNOS complex may be involved in this process, as indicated in our study by a decrease in release of nitrite in the coronary effluent in the diabetic heart and its significant attenuation by daidzein pre-treatment. Roth and Patel (69) demonstrated that interaction of signalling molecules with caveolae is necessary for cardiac protection. The results obtained in our study support this viewpoint.

In the present study, we have used daidzein as an inhibitor of expression of caveolin in male as well as female rats. A limitation of daidzein, is that being a phytoesterogen it may modulate the estrogens status in female animals. However, in an earlier study, no significant difference in the effect of daidzein was detected between male and female rats (data not shown). Also, the restoration of the cardioprotective effect of IPC in diabetic rat heart by combination of daidzein and sodium nitrite was not greater than that observed when these drugs were used alone, suggesting thereby that these two drugs act by the same mechanism i.e., NO pathway.

Conclusions

On the basis of above discussion, it may be concluded that attenuation of cardioprotective effect of ischemic preconditioning in diabetic rat heart is due to some defect in caveolin-eNOS complex in diabetic heart, which leads to, a decrease in the availability of NO and the consequent decreased activation of mito K_{ATP} channels. Also, the IPC-induced changes in eNOS and NO in daidzein pre-treated diabetic heart, closely mimic those produced by IPC in the non-diseased heart.

Limitation of the present study

Ideally, the proposed caveolin-eNOS interaction should have been assessed by coimmunoprecipitation study or by caveolin isolation.

Authors' contributions
All authors, except MS approved the final manuscript. PA did most of the experimental work and data acquisition under the supervision of HNY, MS and PLS. HNY and PLS did the data analysis, interpretation and writing of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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