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Role of Heme-Oxygenase-1 in Attenuated Cardioprotective Effect of Ischemic Preconditioning in Hyperlipidemic Myocardium

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Abstract- Nitric Oxide (NO) is responsible for cardioprotective effect of ischemic preconditioning (IPC). Heme Oxygenase-1 (HO-1) facilitates release of NO by disrupting caveolin-eNOS complex. Both, the expression/activity of HO-1 and the IPC mediated cardioprotection are decreased significantly in hyperlipidemia. In this study the role of HO-1 in attenuation of IPC- induced cardioprotective effect in hyperlipidemic rat was investigated. Hyperlipidemia was induced by feeding high fat diet to Wistar rats. Isolated Langendorff heart preparation model was used. Cardioprotective effect was assessed by myocardial infarct size measurement and release of Lactate Dehydrogenase (LDH), Creatine Kinase (CK-MB) in coronary effluent. Nitrite estimation was done to indirectly infer the level of cardiac NO production. In hyperlipidemic rat, IPC-induced cardioprotection and release of NO were significantly decreased. Perfusion with sodium nitrite (NO precursor) and pre-treatment with daidzein (DDZ) (caveolin inhibitor) and hemin (HO-1 inducer), alone or in combination significantly restored the attenuated cardioprotective effect of IPC in hyperlipidemic rats. Administration of zinc protoporphyrin (ZnPP), HO-1 inhibitor, significantly abolished the observed cardioprotection in hemin pre-treated hyperlipidemic rat. The significant restoration of the attenuated cardioprotective effect of IPC following induction of HO-1 by hemin in hyperlipidemia was observed. The results indicated that attenuation of IPC-induced cardioprotective effect may be due to the decrease in HO-1 induced NO release in hyperlipidemic rat heart.

Keywords: heme oxygenase-1, ischemic preconditioning, hyperlipidemic rat heart, hemin, daidzein.

1. INTRODUCTION

Coronary artery disease is a leading cause of morbidity and mortality worldwide [1, 2]. Inadequate blood flow to the myocardium leads to ischemia and, early reperfusion is necessary for the viability of myocardium [3]. Reperfusion after a prolonged period of ischemia is not without risk, it damages the myocardium, which is known as ischemia-reperfusion injury [4, 5]. Ischemic preconditioning (IPC), is a powerful endogenous cardioprotective phenomenon in which short intermittent cycles of sublethal ischemia, followed by reperfusion before the

subsequent prolonged ischemic insult, improves the tolerance against ischemia-reperfusion-induced injury [6, 7]. IPC mediated cardioprotection has been documented in various species including human beings [7, 8].

IPC produces cardioprotection by stimulating the generation of various endogenous ligands which bind to their respective G-protein coupled receptors [9, 10] and initiate a signalling cascade i.e., activation of PI-3K/Akt [11], phosphorylation of eNOS, generation of NO and by opening of mito K_{ATP} channel [12, 13]. The cardioprotective effect of IPC is attenuated in hyperlipidemic myocardium and it may be due to decreased HO-1 [14, 15], impairment of K_{ATP} channel [16] impairment of PI-3K/AKT pathway [17, 18] and altered activation of JAK/STAT and MAPK, GSK-3 β [19, 20]. Hence, the mechanism involved in attenuation of cardioprotective effect of IPC in hyperlipidemic myocardium, remain to be elucidated.

Caveolae are the specialised membrane domains which serve as organizing centres for cellular signal transduction [21]. Various signalling molecules like src-like kinases, tyrosine kinase, members of Ras-MAPK cascade and eNOS [22] are localized within caveolae. Caveolin is also a well known negative regulator of eNOS and these results in decreased availability of NO [23, 24] which is responsible for cardioprotective effect of IPC [13]. It has been reported that expression of caveolin is upregulated in hyperlipidemic myocardium [25].

Heme-oxygenase is the rate-limiting enzyme in the biochemical pathway responsible for catabolism of heme into ferrous (Fe^{++}) ion, carbon monoxide, and biliverdin, the latter being subsequently converted into bilirubin by biliverdin reductase [26]. HO-1 is localized in the membrane caveolae and the inner leaflet of the plasma membrane where it interacts with caveolin [27]. In transgenic mice, the overexpression of Heme-oxygenase-1, conversely regulates the expression of caveolin [25]. Moreover, HO-1 facilitates release of NO by disrupting association of caveolin with eNOS [25]. It has been reported that a decrease in the cardiospecific expression of HO-1 exacerbates the ischemia reperfusion-induced injury [26], while upregulation of HO-1 produces cardioprotection against ischemia-reperfusion induced injury [27]. Transgenic mice

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expressing cardiac-specific HO-1 are resistant, while the heart of HO-1 knock-out mice is more susceptible to ischemia-reperfusion-induced injury [28]. In hyperlipidemia, the expression and activity of HO-1 is reduced [29] whereas the increase in HO-1 in hyperlipidemic rats is associated with activated eNOS [30]. Therefore, the present study was designed to investigate the role of Heme- Oxygenase-1 in attenuated cardioprotective effect of IPC in hyperlipidemic rat hearts.

II. MATERIALS AND METHODS

Daidzein (0.2mg/Kg/s.c) (Enzo Life Sciences International, Inc., USA) was dissolved in 10% Dimethyl Sulphoxide (DMSO) and then injected to the animals for 7 days after 8 weeks of high fat diet administration. Hemin (4mg/kg/i.p.) (Himedia Laboratories Pvt. Ltd., Mumbai) was dissolved in 0.2M NaOH and was injected 18 h before isolation of heart. Zinc Protoporphyrin (50µg/kg/i.p.) (Enzo Life Sciences International, Inc., USA) was dissolved in DMSO and injected 6 hr before hemin treatment [31]. TTC Stain, Tris-chloride buffer, sulphanilamide, phosphoric acid and sodium nitrite was purchased from CDH Pvt. Ltd., New Delhi. N-(1-Naphthyl) ethylenediamine dihydrochloride was purchased from Himedia Laboratories Pvt. Ltd., Mumbai. The LDH enzymatic estimation kit and CK-MB enzymatic estimation kit was purchased from Coral Clinical Systems, Goa, India. All other reagents used in this study were of analytical grade and always freshly prepared before use.

a) Animals

Age matched young male Wistar rats, weighing 180-250 g housed in animal house and provided 12 h light and 12 h dark cycle were used. They were fed on standard chow diet (Ashirwad Industries Ltd., Ropar, India) and provided water ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee in accordance with the National (CPCSEA) Guidelines on the Use of Laboratory animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

b) Induction of experimental hyperlipidaemia

Male Wistar rats (180-250) were employed in the present study. Experimental hyperlipidemia was induced by high fat diet (corn starch 44.74 g, casein 14 g, sucrose 10 g, butter 20 g, fibre 5 g, mineral mix 3.5 g, vitamin mix 1 g, choline 0.25 g, terbutylhydroquinone 0.0008 g, cholesterol 1 g, cholic acid 0.5 g) for 8 weeks. Serum cholesterol and triglyceride level was estimated spectrophotometrically at 505 nm by PEG and GPO / PAP method (Trinder, 1969; Bucolo, 1973, Fossati, 1982) using enzymatic kits (Coral Clinical Systems, Goa, India). Serum cholesterol level 800- 1000 mg/dl and

serum triglyceride level 200- 300 mg/dl were considered to be hyperlipidemic.

c) 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 [30]. Isolated heart was retrogradely perfused at constant pressure of 80 mmHg with Kreb's-Henseleit buffer (NaCl 118 mM; KCl 4.7 mM; CaCl₂ 2.5 mM; MgSO₄.7H₂O 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 LDH, CK-MB and nitrite release [32].

d) Assessment of myocardial injury

The assessment of myocardial infarct size was done by using triphenyltetrazolium chloride (TTC) staining method. 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 0.2M 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 [33]. LDH and CK-MB were estimated by using commercially available kits. Values of LDH and CK-MB were expressed in international units per litre (IU/L).

e) Nitrite estimation

Nitrite is 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. Nitrite release in coronary effluent was measured. 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 [32].

f) *Experimental design (n= 72; each group contained 6 rats)*

Group 1 Sham control.



Group 2 Diadzein per se



Group 3 Hemin per se.



Group 4 Ischemia reperfusion control.



Group 5 Ischemic preconditioning control.



Group 6 IPC in sodium nitrite perfused normal rat hearts.



Group 7 Ischemic preconditioning in hyperlipidemic rat heart.



Group 8 IPC in sodium nitrite perfused hyperlipidemic rat hearts.



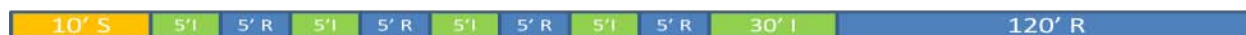
Group 9 IPC in diadzein pretreated hyperlipidemic rat heart.



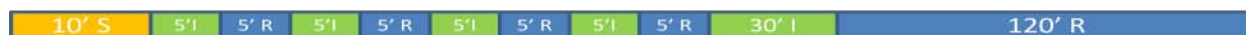
Group 10 IPC in Hemin (4 mg/kg i.p) pretreated Hyperlipidemic rat heart.



Group 11 IPC in Diadzein and Hemin pretreated Hyperlipidemic rat heart.



Group 12 IPC in Zn protoporphyrin (50µg /kg i.p) and hemin (4mg/kg i.p) pretreated hyperlipidemic rat heart.



Diagrammatic representation of experimental protocol is shown. In all groups, isolated rat heart was perfused with K-H (Krebs-Hensleit) solution and allowed for 10 min of stabilization. Isolated rat heart preparation was stabilized for 10 min and then perfused continuously with K-H solution for 190 min.

g) *Data analysis and statistical procedures*

All values were expressed as mean $\pm$ standard deviation (S.D). Statistical analysis was performed using Graphpad Prism Software (5.0). The data obtained from the various groups were statistically analysed using student t-test, one-way analysis of variance (ANOVA), two way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $P < 0.05$ was considered to be statistically significant.

III. RESULTS

a) Effect of high fat diet on body weight

The high fat diet was fed for 8 weeks to the rats and a significant increase in body weight was observed as compared to basal value (Fig. 1).

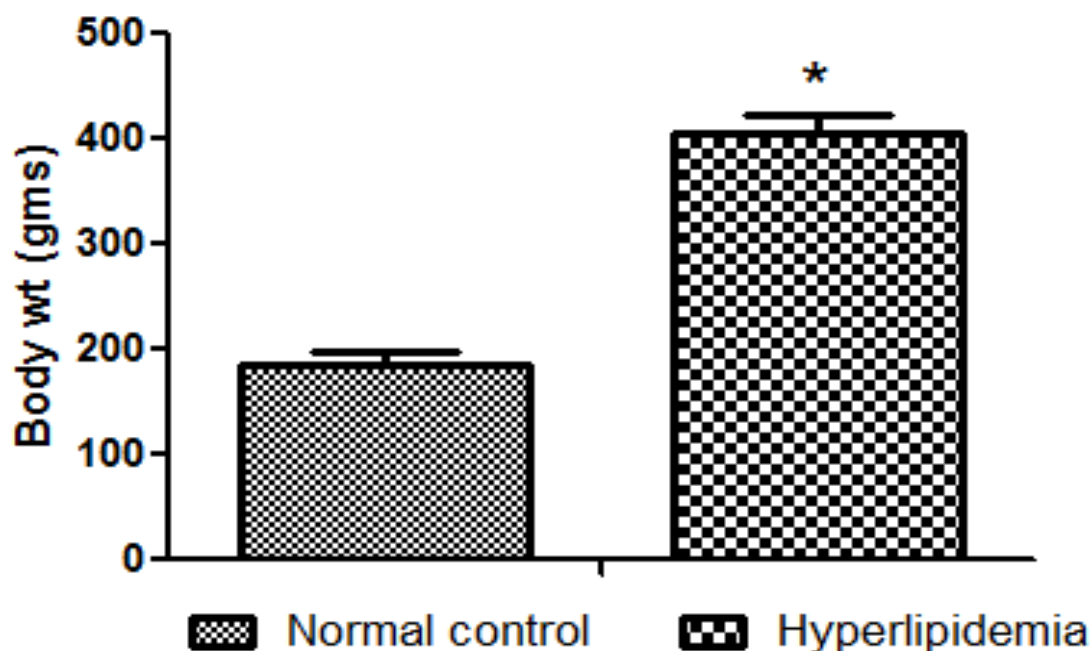


Fig. 1: Effect of high fat diet administration on serum body weights of rats.

Values were expressed as mean $\pm$ S.D.

b) Effect of high fat diet on serum cholesterol and triglyceride level

After feeding high fat diet for 8 weeks to the rats, serum cholesterol and serum triglycerides level was estimated and a significant increase in serum cholesterol and serum triglyceride level was observed as compared to basal value. (Fig. 2)

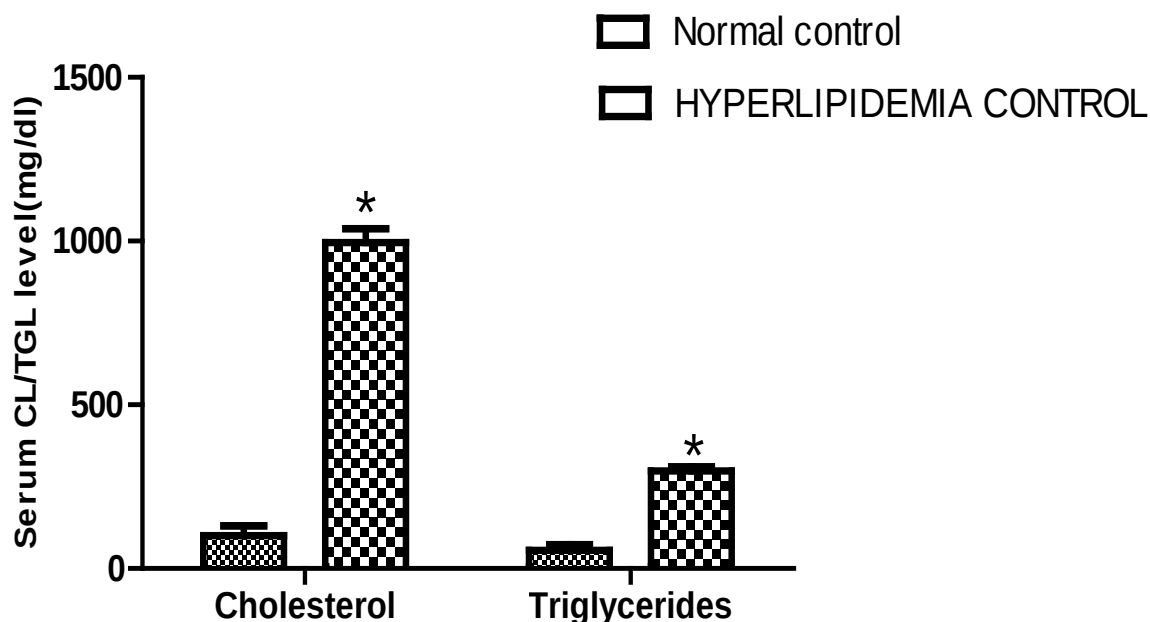


Fig. 2: Comparison of serum cholesterol and triglyceride level in normal and hyperlipidemic rat hearts.

Values are expressed as mean $\pm$ S.D.

c) *Effect of ischemic preconditioning and pharmacological interventions on myocardial injury (Infarct size, LDH and CK-MB)*

Global ischemia for 30 min followed by 120 min of reperfusion significantly increased the myocardial injury as compared to sham control. Four episodes of IPC significantly decreased I/R-induced increase in myocardial injury in normal rat heart. However, ischemic preconditioning failed to decrease the myocardial injury

in hyperlipidemic rat heart. Moreover, IPC induced decrease of myocardial injury was significantly restored in sodium nitrite perfused hyperlipidemic rat heart. Similar results were obtained in daidzein and hemin, administered alone or in combination. Administration of ZnPP significantly blocked the observed cardio-protective effect in hemin in hyperlipidemic rat (Fig. 3, 4, 5).

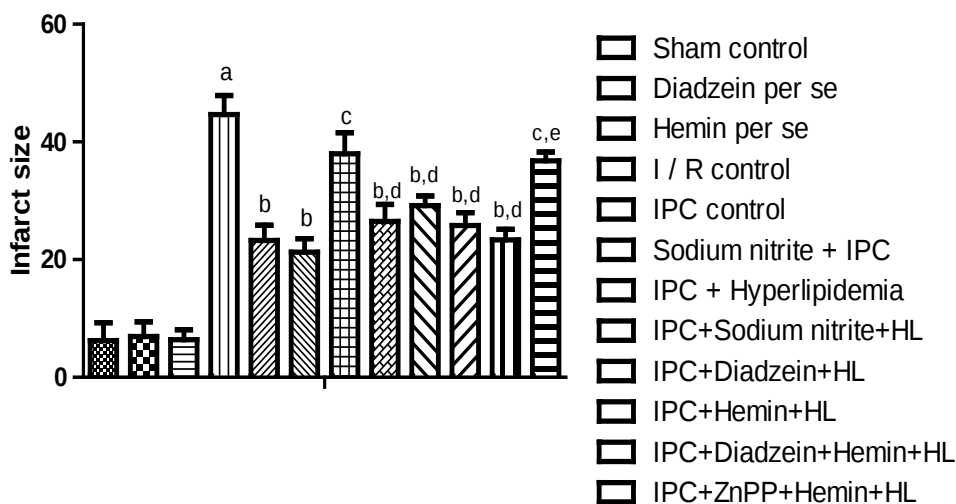


Fig. 3: Effect of pharmacological interventions on myocardial infarct size.

Values are expressed as mean $\pm$ S.D. a = $p < 0.05$ vs. Sham Control and basal value; b = $p < 0.05$ vs. I/R Control; c = $p < 0.05$ vs. IPC control; d = $p < 0.05$ vs. IPC in hyperlipidemic rat heart; e = $p < 0.05$ vs. IPC in hemin treated hyperlipidemic rat heart.

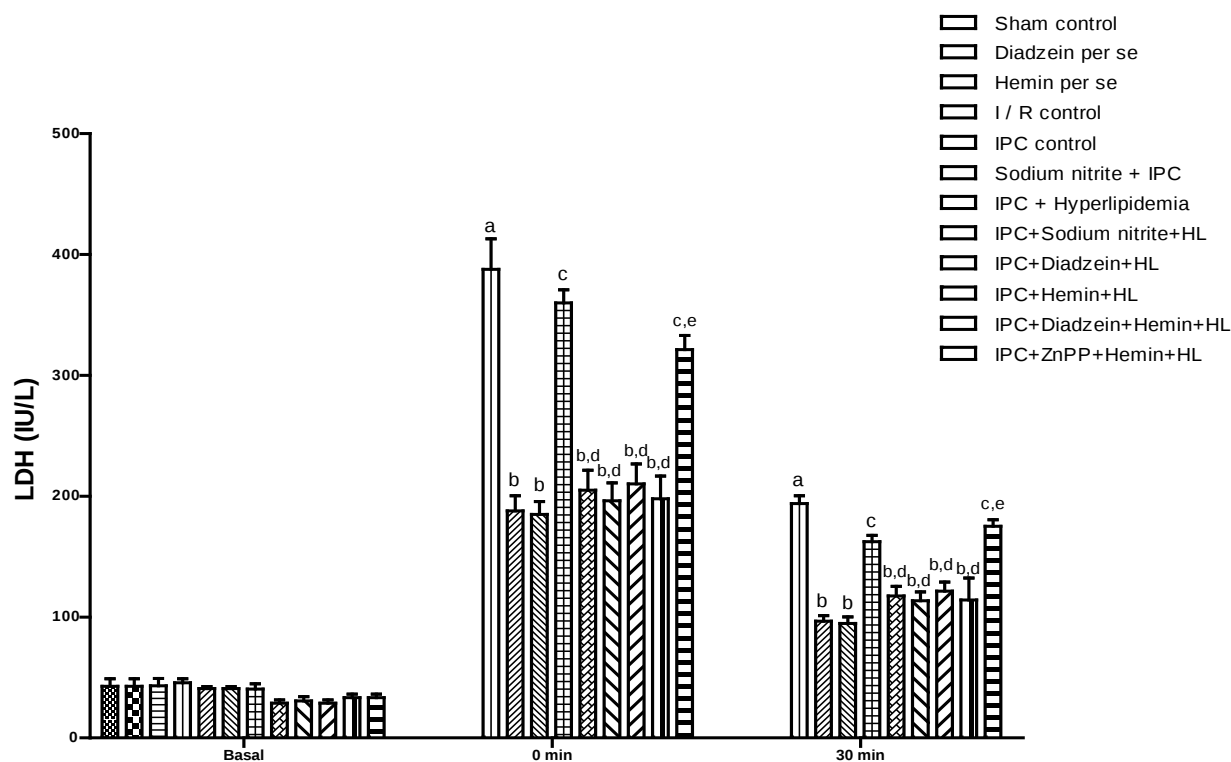


Fig. 4: Effect of pharmacological interventions on myocardial release of lactate dehydrogenase (LDH).

Values are expressed as mean $\pm$ S.D. a = $p < 0.05$ vs. Sham Control and basal value; b = $p < 0.05$ vs. I/R Control; c = $p < 0.05$ vs. IPC control; d = $p < 0.05$ vs. IPC in hyperlipidemic rat heart; e = $p < 0.05$ vs. IPC in hemin treated hyperlipidemic rat heart.

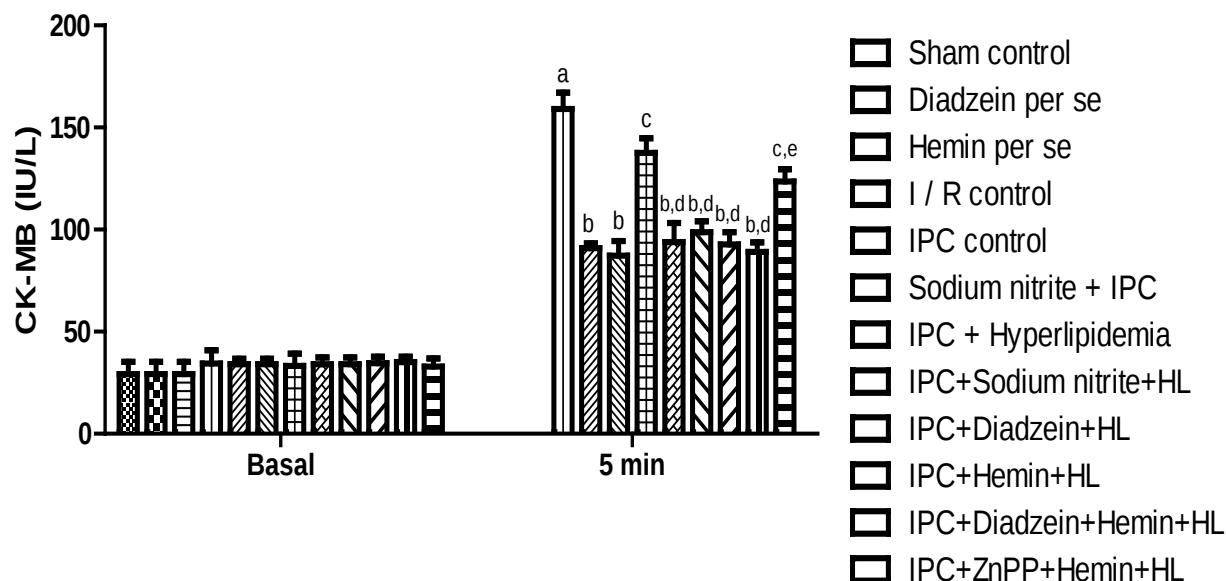


Fig. 5: Effect of pharmacological interventions on myocardial release of Creatine Kinase (CK-MB).

Values are expressed as mean $\pm$ S.D. a = $p < 0.05$ vs. Sham Control and basal value; b = $p < 0.05$ vs. I/R Control; c = $p < 0.05$ vs. IPC control; d = $p < 0.05$ vs. IPC in hyperlipidemic rat heart; e = $p < 0.05$ vs. IPC in hemin treated hyperlipidemic rat heart.

d) *Effect of ischemic preconditioning and pharmacological interventions on the nitrite release in coronary effluent*

Global ischemia for 30 min followed by 120 min of reperfusion significantly decreased the nitrite release. Four episodes of IPC significantly restored the I/R induced decrease in nitrite release in normal rat heart. However, ischemic preconditioning failed to increase the

nitrite release in hyperlipidemic rat heart. Moreover, IPC induced increase of nitrite release was significantly restored in sodium nitrite perfused hyperlipidemic rat heart. Pre-treatment with daidzein and hemin, alone or in combination also restored it. Furthermore, administration of ZnPP significantly abolished the restored cardioprotective effect of hemin in hyperlipidemic rat (Fig.6).

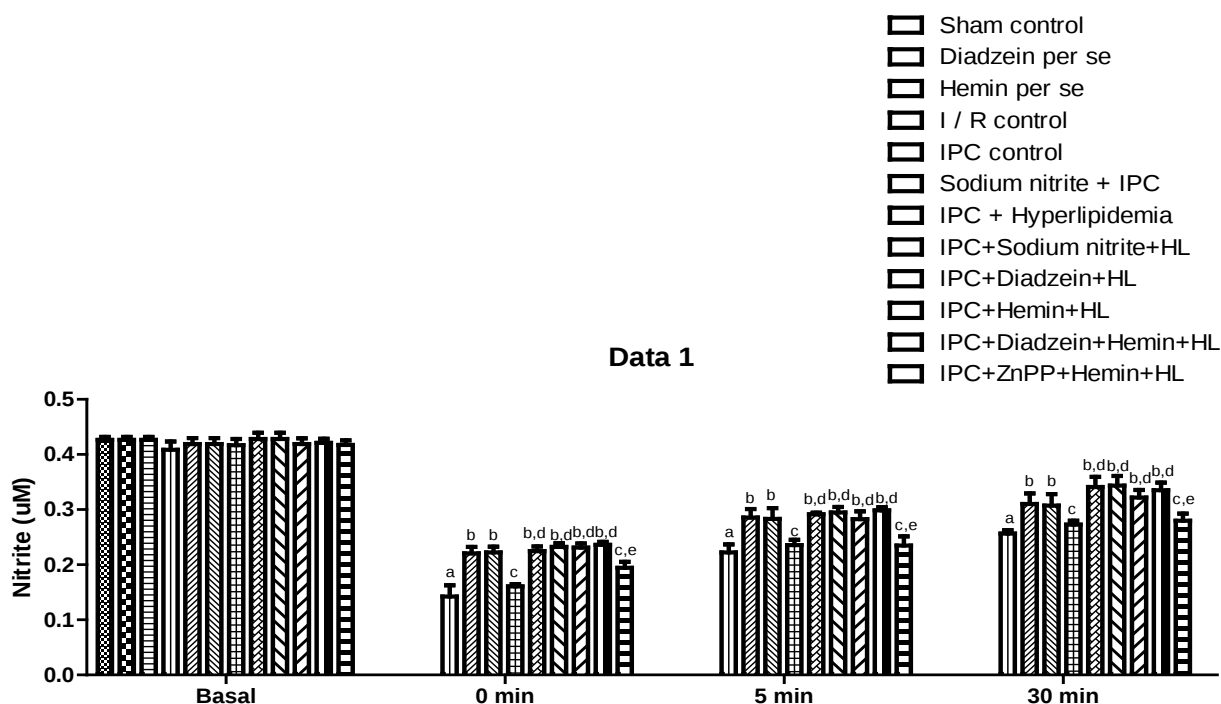


Fig. 6: Effect of pharmacological interventions on myocardial release of nitrite.

Values are expressed as mean $\pm$ S.D. a = $p < 0.05$ vs. Sham Control and basal value; b = $p < 0.05$ vs. I/R Control; c = $p < 0.05$ vs. IPC control; d = $p < 0.05$ vs. IPC in hyperlipidemic rat heart; e = $p < 0.05$ vs. IPC in hemin treated hyperlipidemic rat heart.

IV. DISCUSSION

This study was designed to investigate the role of HO-1 in attenuated cardioprotective effect of IPC in hyperlipidemic rat hearts. After a prolonged period of ischemia, reperfusion produces further damage to myocardium which is known as ischemia reperfusion injury. The ischemic preconditioning induced by four episodes of 5 min global ischemia and 5 min reperfusion was reported to produce cardioprotective effect in isolated rat heart preparation [34]. Our findings were in agreement with these phenomenon's. The cardioprotective effect of IPC had been reported to be significantly attenuated in hyperlipidemia. Our results were in accordance with these published studies [35].

Perfusion of sodium nitrite (NO donor) produces cardioprotection in isolated heart from normal rat, subjected to global ischemia [36]. In our study, perfusion of sodium nitrite in isolated hyperlipidemic rat heart followed by IPC, significantly restored the attenuated effect of IPC in diabetic myocardium.

Release of nitric oxide during the ischemic preconditioning was reported to produce cardioprotection against ischemia-reperfusion induced injury [12]. In our study, IPC significantly increased the release of NO (measured in coronary effluent), as compared to ischemia reperfusion control group. However, this IPC mediated increase in release of nitric oxide was significantly decreased in hyperlipidemic rat heart. Sodium nitrite perfusion in hyperlipidemic rat heart significantly restored the attenuated cardioprotective effect of ischemic preconditioning. Thus, the reduced release of NO in hyperlipidemic rat heart may be responsible for attenuation of cardioprotection mediated by IPC in hyperlipidemic rat. It was interesting to note that treatment with sodium nitrite did not enhance the cardioprotective effect of IPC in normal rat heart. This indicated that once IPC mediated increased generation of NO achieved the threshold for cardioprotection; addition of sodium nitrite was unable to further increase the myocardial protection by IPC.

Caveolae are 50-100 nm invaginated plasma membrane domains which serve as organizing centers of signal transduction [37]. Caveolins are proteins that form the structure of caveolar membrane, act as signalosomes for GPCR and other molecules such as NOS and Src-like kinases [38]. Increased expression of caveolin, leads to the, decreased phosphorylation of endothelial nitric oxide synthase and consequent decreased generation of nitric oxide. Further, it has been reported that expression of caveolin is upregulated in hyperlipidemic myocardium [39]. Thus, it may results in increased formation of Caveolin-eNOS complex, which decreases the availability of nitric oxide. It has been reported that NO is responsible for cardioprotective effect of ischemic preconditioning [40].

Upregulation of caveolin in diabetic rat heart may inhibit the activity of eNOS by making its complex which leads to a decrease in the release of NO [41]. Administration of daidzein increases the generation of nitric oxide by inhibiting the caveolin-eNOS complex and subsequent activation of the eNOS [42]. In our study, one week of pretreatment of hyperlipidemic rat with daidzein, a caveolin inhibitor [42], significantly restored the cardioprotective effect of ischemic preconditioning in hyperlipidemic rat heart, noted in terms of decrease in infarct size and release of LDH, CKMB, and also increase in the release of NO. Our findings were in agreement with reports from other laboratories [20].

Heme-Oxygenase-1 is localized in the membrane caveolae of the plasma membrane where it is interacts with caveolin [27]. It has been reported that a decrease in the cardiospecific expression of HO-1 exacerbates while an upregulation of HO-1 produces cardioprotection against ischemia-reperfusion injury [43]. HO-1 facilitates release of NO by disrupting complex of caveolin and eNOS [43]. The expression of HO-1 is diminished into hyperlipidemic myocardium. In our study, pretreatment with hemin, a heme-oxygenase-1 inducer, restored the decrease in release of nitric oxide and significantly restore the attenuated cardioprotective effect of ischemic preconditioning in hyperlipidemic rat heart.

Thus it was speculated that the attenuated cardioprotective effect of IPC in hyperlipidemic rat heart may be due to inhibition of eNOS by enhancing the binding of eNOS with caveolin, which leads to decrease in the release of nitric oxide. Also, administration of ZnPP, an inhibitor of HO-1, significantly blocked the observed cardioprotection and increase in release of NO in hearts of hemin pretreated hyperlipidemic rats. Furthermore, the restoration of the attenuated cardioprotective effect of IPC in hyperlipidemic rat heart by combination of daidzein and hemin was not greater than that observed when the drugs were administered alone. This suggested that these two drugs may be acting via the same mechanism i.e., NO pathway.

On the basis of above discussion it was clear that activation of heme-oxygenase-1 enzyme, by a specific inducer i.e. hemin, restored the cardioprotective effect of ischemic preconditioning in hyperlipidemic rat heart, by disrupting the caveolin-eNOS complex and there by enhancing the release of NO. Further, pretreatment with ZnPP, a specific heme-oxygenase-1 inhibitor, significantly blocked the restoration of cardioprotective effect of ischemic preconditioning in hemin pretreated hyperlipidemic rat heart. Therefore, it was concluded that attenuation of cardioprotective effect of ischemic preconditioning in hyperlipidemic rat heart, was due to impairment of HO-1 induced release of nitric oxide.

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Conflict of Interest: The authors declared no conflict of interest.

Abbreviations:

NO, Nitric Oxide;
IPC, ischemic preconditioning;
eNOS, endothelial nitric oxide synthase;
HO-1, Heme Oxygenase-1;
LDH, Lactate Dehydrogenase;
CK-MB, Creatine Kinase;
DDZ, Daidzein;
ZnPP, Zinc protoporphyrin;
K-H, Krebs-Henseleit

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