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Possible role of clofibrate in attenuated cardioprotective potential of ischemic preconditioning in hypercholesterolaemic rat hearts

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ABSTRACT

The present study has been designed to investigate the role Clofibrate in hypercholesterolaemia-induced attenuation of cardioprotective effect of ischemic preconditioning. Experimental hypercholesterolaemia was produced by feeding high fat diet to rats for a period of 28 days. Isolated langendorff's perfused normal and hypercholesterolaemic rat hearts were subjected to global ischemia for 30 min followed by reperfusion for 120 min. The myocardial infarct size was assessed macroscopically using triphenyltetrazolium chloride staining. Coronary effluent was analyzed for lactate dehydrogenase and creatine kinase release to assess the extent of cardiac injury. Moreover, the oxidative stress in heart was assessed by measuring TBARS and GSH. The ischemia-reperfusion has been noted to induce oxidative stress by increasing TBARS, superoxide anion generation and decreasing reduced form of glutathione in normal and hypercholesterolaemic rat hearts. Moreover, I/R produced myocardial injury, which was assessed in terms of increase in myocardial infarct size, LDH and CK-MB release in coronary effluent and decrease in coronary flow rate in normal and hypercholesterolaemic rat hearts. In count, the hypercholesterolaemic rat heart showed enhanced I/R-induced myocardial injury with high degree of oxidative stress as compared with normal rat hearts subjected to I/R. Four episodes of IPC afforded cardioprotection against I/R-induced myocardial injury in normal rat hearts as assessed in terms of improvement in coronary flow rate and reduction in myocardial infarct size, LDH, CK-MB and oxidative stress. On the other hand, IPC mediated myocardial protection against I/R-injury was abolished in hypercholesterolaemic rat hearts may be due to consequent down-regulation of PPAR-α with high oxidative stress. Treatment with Clofibrate (300mg/kg/day, i.p.), an activator of PPAR-α has not affected the cardioprotective effects of IPC in normal rat hearts, but its treatment markedly restored the cardioprotective potential of IPC in hypercholesterolaemic rat hearts.

Key words: Hypercholesterolaemia, Ischemia-reperfusion injury, Ischemic preconditioning, Clofibrate, PPAR-α.

INTRODUCTION

Coronary artery disease is the leading cause of morbidity and mortality and its prevalence is continuously increasing worldwide [1]. Myocardial ischemia is a condition in which heart tissue gets inadequate blood flow followed by inadequate oxygen and nutrient supply. The restoration of coronary blood flow to an ischemic myocardium is

mandatory in order to avoid the myocardial damage. However, reperfusion of the previously ischemic myocardium is often followed by detrimental changes in myocardial tissues, is known as ischemia-reperfusion (I/R) injury [2]. Brief episodes of ischemia and reperfusion render the heart more tolerant to subsequent sustained ischemia and reperfusion, known as ischemic preconditioning (IPC) [3,4]. IPC has been noted to reduce I/R-induced myocardial injury by decreasing oxidative stress, limiting myocardial infarct size, decreasing neutrophill (PMN) accumulation, preserving coronary endothelial function and inhibiting apoptosis and necrosis [4-7]. Various mechanisms involved in the cardioprotective potential of IPC include activation of phosphatidylinositol-3-kinase (PI3K)/Akt pathway, generation of nitric oxide (NO), activation of mitochondrial ATP-sensitive K^+ channels (mito K_{ATP} channels) and closure of mitochondrial permeability transition pore (MPTP) [4, 8-9]. However, the cardioprotective and infarct size limiting effect of IPC has been abolished in some pathological conditions such as diabetes, obesity, heart failure, hypercholesterolaemia, ageing and hypertension [10-12]. Hypercholesterolaemia (Hcl), a condition of elevated level of Cholesterol, lipids and triglycerides in blood, has been considered to be an independent risk factor for cardiovascular diseases [13-14]. Hcl has been shown to generate high amount of reactive oxygen species (ROS) by activating NADPH oxidase [14-16]. It has been recently reported that Hcl decreased the eNOS mRNA expression followed by increased oxidative stress and decreased bioavailability of NO occur to damage the vascular endothelium [17]. We have noted that the cardioprotective potential of IPC was abolished in the hypercholesterolaemic rat's hearts. However, the mechanism involved in the attenuation of cardioprotective effect of IPC in the hypercholesterolaemic rat heart is not known. We have noted that the hypercholesterolaemic rat heart produced high degree of oxidative stress upon reperfusion when compared with the normal rat heart subjected to I/R. Thus, it was believed that the signaling mechanisms activated by high degree of oxidative stress may play a detrimental role in the attenuation of cardioprotective effect of IPC in the hypercholesterolaemic rat heart.

Peroxisome proliferator activated receptor-α (PPAR-α) is a subfamily of the nuclear receptor superfamily naturally activated by ligands such as free fatty acids and eicosanoids [18]. PPARs are ligand-activated transcriptional factors that regulate genes important in cell differentiation and various metabolic processes, especially lipid and glucose homeostasis. It has been reported that PPAR-α gets down regulated during high amount of oxidative stress [19-20]. Further, PPAR-α has been noted to activate PI3K/Akt pathway and activation of PI3K/Akt pathway has been previously well demonstrated to be involved in the cardioprotective effect of IPC [21-22]. Moreover, PPAR-α down regulation has been implicated in the pathogenesis of I/R-induced myocardial injury. Clofibrate (Clo) has been shown to be a selective activator of PPAR-α [23]. Therefore, the present study has been designed to investigate the possible effects of Clofibrate, an activator of PPAR-α, in the abrogated cardioprotective effect of IPC in hypercholesterolaemic rat hearts subjected to I/R.

MATERIALS AND METHODS

Animals

Wistar albino rats of either sex weighing about 180-220 g were employed in the present study. They were fed on standard chow diet (Ashirwad Industries Private Ltd., Punjab, India) and were provided water *ad libitum*. They were housed in departmental animal house and were exposed to 12 h light and dark cycles. All animals were maintained as per the guidelines for the care and use of laboratory animals. The study protocol was approved by Institutional Animal Ethics Committee (CPCSEA/IAEC/05/2011).

Diet-Induced Hypercholesterolaemia

Experimental hypercholesterolaemia was produced by feeding high fat diet (casein, 200 g; coconut oil, 250 g; cholesterol, 10 g; cholic acid, 5 g; sucrose, 484 g; choline chloride, 2 g; DL-methionine, 4 g; vitamin mix, 10 g; mineral mix, 35 g were added to make 1.0 kg of diet) to rats for a period of 28 days [24]. Mineral mix was composed of NaCl, 5.57 g; KCl, 32 mg; $MgSO_4$, 2.29 g; $FeSO_4.7H_2O$, 108 g; $CaHPO_4$, 70 mg; $CuSO_4.5H_2O$, 0.1 mg; $MnSO_4.H_2O$, 0.01 mg; $ZnSO_4.H_2O$, 28.7 mg; KI, 0.025 mg; $COCl_2.6H_2O$, 9 mg and MgO, 0.15 mg. Moreover, vitamin mix was comprised of retinol acetate, 5000 IU; cholecalciferol, 400 IU; 7-dehydrocholesterol, 2000 IU; tocopheryl acetate, 15 mg; thiamine hydrochloride, 5 mg; riboflavin, 5 mg; nicotinamide, 45 mg; D-panthenol, 5 mg; pyridoxine hydrochloride, 2 mg; ascorbic acid, 75 mg; folic acid, 1000 µg and cyanocobalamin, 5 µg.

Assessment of Diet-Induced Hypercholesterolaemia

Hypercholesterolaemia was determined by estimating the levels of Total cholesterol, High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), Very Low Density Lipoprotein (VLDL) and Triglycerides in blood serum using commercially available kits. Values were expressed in mg/dl.

Estimation of Serum Total Cholesterol and High Density Lipoprotein (HDL) Levels
Serum total cholesterol and HDL levels were estimated spectrophotometrically (UV1 Spectrophotometer, Thermo Electron Corporation, England) at 505 nm by the method of Allain et al. (1974) using commercially available kit (Monozyme India Ltd., Secunderabad, India).

Total Cholesterol Level
Serum Total Cholesterol = Abs. of Cholesterol Test / Abs. of Standard x 200

HDL Level
Serum HDL level = Abs. of HDL Test / Abs. of Standard x 50

Estimation of Serum Triglyceride Levels
Serum triglycerides were estimated spectrophotometrically (UV1 Spectrophotometer, Thermo Electron Corporation, England) at 546 nm by enzymatic glycerol phosphate oxidase/peroxidase (GPO/POD) method (Werner et al., 1981) using commercially available kit (Kamineni Life Sciences Private Ltd., Hyderabad, India).

Triglyceride Level
Serum Triglyceride levels (mg/dl) = Abs. of Test / Abs. of Standard x 200

Estimation of Very Low Density Lipoprotein (VLDL) and Low Density Lipoprotein (LDL) Levels
VLDL and LDL concentrations were calculated from the Friedewald equation [25].

VLDL Level
Serum VLDL levels (mg/dl) = Triglyceride level/ 5, and

LDL Level
Serum LDL levels (mg/dl) = Total cholesterol-(HDL level + VLDL level).

Isolated rat heart preparation
Heparin (500U *i.p.*) was administered about 20 min before sacrificing the animal by cervical dislocation. The heart was rapidly excised and immediately mounted on Langendorff apparatus [26]. The heart was enclosed in a double walled jacket and the temperature of which was maintained at 37°C by circulating warm water. The preparation was perfused with Krebs Henseleit (K-H) solution (NaCl 118 mM; KCL 4.7 mM; $CaCl_2$ 2.5 mM; $MgSO_4.7H_2O$ 1.2 mM; $NaHCO_3$ 25mM; KH_2PO_4 1.2 mM; $C_6H_{12}O_6$ 1 mM) of pH 7.4, maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. The coronary flow rate was maintained at around 7 ml/min by keeping the perfusion pressure at 80 mmHg. Global ischemia was produced for 30 min by blocking the inflow of physiological solution and it was followed by reperfusion of 120 min after 10 min of stabilization. The coronary flow rate was noted at basal (before global ischemia), 0 min (at the onset of reperfusion), 5 min, 30 min and 120 min of reperfusion.

Ischemic Preconditioning
Langendorff's perfused normal and hypercholesterolaemic hearts were subjected to four episodes of ischemia after 10 min of stabilization followed by reperfusion, each comprising of 5 min occlusion and 5 min reperfusion, than 30 min of global ischemia followed by reperfusion for 120 min to produce ischemic preconditioning.

Assessment of myocardial injury
The I/R-induced myocardial injury was assessed by estimating the release of lactate dehydrogenase (LDH) and creatine kinase (CK-MB) in the coronary effluent and measuring the infarct size in the heart.

Estimation of LDH and CK-MB
The myocardial injury was assessed by measuring the release of LDH and CK-MB in the coronary effluent using the commercially available enzymatic kits (Vital Diagnostics, Thane, Maharastra, India). LDH was measured in the coronary effluent by UV-Kinetic method, which is based on the principle that LDH catalyses the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. LDH activity is proportional to increase in absorbance due to reduction of NAD. The LDH activity is expressed in U/L using the formula: LDH activity (U/L) = $\Delta A/min \times 3376$. Ck-MB was measured in the coronary effluent by immune-inhibition method, which is based on the principle that CK-M fraction of CK-MM in the sample is completely inhibited by CK-M antibody

present in the reagent. Then the activity of CK-B fraction is measured and the CK-MB activity is expressed in U/L using the formula: CK-MB activity (U/L) = ΔA/min × 6752.

Infarct size measurement

Hearts were removed from Langendorff's apparatus. Both auricles, root of aorta and right ventricle were excised and left ventricle was kept overnight at -4°C. Frozen ventricle was sliced into uniform sections of 2-3 mm in thickness. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) solution in 0.1 M tris buffer, of pH 7.8, for 20 min at 37°C. TTC stain reacts with dehydrogenase enzyme in the presence of cofactor NADH to form formazon pigment in viable cells, which is brick red in colour. The infarcted cell that has lost dehydrogenase enzyme remains unstained. Thus, the infarcted portion of the myocardium remains unstained while the normal viable myocardium is stained brick red with TTC. Infarct size was measured macroscopically using volume method [27].

Assessment of oxidative stress

The left ventricle was minced and homogenized in 0.05 M ice cold phosphate buffer (pH 7.4) using a Teflon homogenizer. The clear supernatant of homogenate was used to estimate thiobarbituric acid reactive substance (TBARS) and reduced form of glutathione (GSH).

Estimation of TBARS

The quantitative measurement of TBARS, an index of lipid peroxidation in heart was performed according to the method of Ohkawa et al. (1979). 0.2 ml of the supernatant homogenate was pipetted out in a test tube, followed by addition of 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 30% acetic acid (pH 3.5) and 1.5 ml of 0.8% of thiobarbituric acid and the volume was made up to 4 ml with distilled water. The test tubes were incubated for 1 hour at 95°C, then cooled and added 1 ml of distilled water followed by addition of 5 ml of n-butanol-pyridine mixture (15:1 v/v). The test tubes were centrifuged at 4000 g for 10 min. The absorbance of developed pink color was measured spectrophotometrically (Thermo Double Beam Spectrophotometer, Thermo Electron Corporation, United Kingdom) at 532 nm. A standard calibration curve was prepared using 1-10 nM of 1,1,3,3-tetramethoxy propane. The concentration of TBARS value was expressed as nanomoles per gm of wet tissue weight [28].

Estimation of superoxide anion generation

The heart was cut into transverse sections and placed in 5 ml of K-H solution buffer containing 100 µM of nitroblutetrazolium (NBT) and incubated at 37ºC for 1.5 hours. NBT reduction was stopped by adding 5 ml of 0.5 N HCL. The heart was minced and homogenized in a mixture of 0.1 N NaOH and 0.1% SDS in water containing 40 mg/l di-ethylene triamine pentaacetic acid (DTPA). The mixture was centrifuged at 20000 g for 20 min and the resultant pellets were resuspended in 1.5 ml of pyridine and kept at 80ºC for 1.5 hours to extract formazon. The mixture was centrifuged at 10000 g for 10 min and the absorbance of formazon was determined spectrophotometrically (UV1 Spectrophotometer, Thermo Electron Corporation, England) at 540 nm. The amount of reduced NBT was calculated using the following formula: Amount of reduced NBT=A.V/(T.Wt.ε.l), where A is absorbance, V is volume of solution (1.5 ml), T is time for which the rings were incubated with NBT (90 min), Wt is blotted wet weight of heart, ε is extinction coefficient (0.72 L/mM/mm) and l is the length of light path (10 mm). Results were expressed as reduced NBT in picomoles per min per mg of wet tissue [29].

Estimation of reduced glutathione

The reduced glutathione (GSH) content in heart was estimated using method of Beutler et al. (1963). The supernatant of homogenate was mixed with trichloroacetic acid (10% w/v) in 1:1 ratio. The tubes were centrifuged at 1000 g for 10 min at 4°C. The supernatant obtained (0.5 ml) was mixed with 2 ml of 0.3 M disodium hydrogen phosphate. Then 0.25 ml of 0.001 M freshly prepared DTNB [(5,5'-dithiobis (2-nitrobenzoic acid) dissolved in 1% w/v citric acid] was added and the absorbance was noted spectrophotometrically (UV1 Spectrophotometer, Thermo Electron Corporation, England) at 412 nm. A standard curve was plotted using 5-50 µM of reduced form of glutathione and results were expressed as micromoles of reduced glutathione per mg of wet tissue weight [30].

Experimental protocol

Twelve groups were employed in the present study and each group comprised of eight animals. A diagrammatic representation of experimental protocol is shown in Fig. 1. In all groups, isolated per-fused rat heart was allowed to stabilize for 10 min by per-fusing with K-H solution.

Group I *(Normal Control): Isolated normal rat heart was perfused for 150 min using K-H solution after 10 min of stabilization.*
Group II *(I/R-Control): Isolated normal rat heart after 10 min of stabilization was subjected to 30 min of global ischemia followed by 120 min of reperfusion.*
Group III *(Clofibrate Per se Normal Control): The rat was given Clofibrate (300mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated normal rat heart was perfused for 150 min using K-H solution after 10 min stabilization.*
Group IV *(Ischemic Preconditioned): After 10 min of stabilization, the normal rat heart was subjected to four episodes each comprised of 5 min of global ischemia followed by 5 min of reperfusion to produce IPC. After four episodes of IPC, the heart was subjected to 30 min of global ischemia followed by 120 min of reperfusion.*
Group V *(Clofibrate Treated I/R-Control): The rat was given Clofibrate (300mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated normal rat heart was then subjected to 30 min of global ischemia followed by 120 min of reperfusion after 10 min of stabilization.*
Group VI *(Clofibrate Treated Ischemic Preconditioned): The rat was given Clofibrate (300mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated normal rat heart was subjected to IPC as mentioned in group IV followed by 30 min of global ischemia and 120 min of reperfusion.*
Group VII *(Hcl Control): Isolated hypercholesterolaemic rat heart was perfused for 150 min using K-H solution after 10 min of stabilization.*
Group VIII *(Hcl-I/R Control): Isolated hypercholesterolaemic rat heart was subjected to 30 min of global ischemia followed by 120 min of reperfusion after 10 min of stabilization.*
Group IX *(Clofibrate Per se Hcl-Control): The rat was given Clofibrate (300mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated hypercholesterolaemic rat heart was perfused for 150 min using K-H solution after 10 min stabilization*
Group X *(Hcl-Ischemic Preconditioned): After 10 min of stabilization, the hypercholesterolaemic rat heart was subjected to IPC as mentioned in Group IV. After IPC, the heart was subjected to 30 min of global ischemia followed by 120 min of reperfusion.*
Group XI *(Clofibrate Treated Hcl-I/R Control): The rat was given Clofibrate (300 mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated hypercholesterolaemic rat heart was then subjected to 30 min of global ischemia followed by 120 min of reperfusion after 10 min of stabilization.*
Group XII *(Clofibrate Treated Hcl-Ischemic Preconditioned): The rat was given Clofibrate (300mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated hypercholesterolaemic rat heart was subjected to IPC as mentioned in group IV followed by 30 min of global ischemia and 120 min of reperfusion.*

Statistical analysis

The results were expressed in mean ±S.D. The data obtained from various groups were statistically analyzed using two way ANOVA followed by Tukey's multiple comparison test. The p values of less than 0.05 were considered to be statistically significant.

Drugs and chemicals

Clofibrate was obtained from Ranbaxy Pvt. Ltd. India as ex-gratia samples. The LDH and CK-MB enzymatic estimation kits were purchased from Vital Diagnostics, Thane, Maharastra, India. DTNB and NBT were obtained from Loba Chem, Mumbai, India. 1,1,3,3-tetramethoxy propane and reduced glutathione were procured from Sigma-Aldrich, USA. HDL kits purchased from Monozyme Ltd, Secunderabad, India. Serum Triglyceride kits purchased from Kamineni Life Sciences Private Ltd, Hyderabad, India. TTC stain and High fat diet purchased from Sanjay Biological, Amritsar, Punjab, India. All other reagents used in this study were of analytical grade.

RESULTS

Rat fed with high fat diet for 28 days with oral gavage significantly increased serum concentrations of total cholesterol (272.1±24.4*), triglycerides (258.7±19.7*), LDL (190.86±18.8*), VLDL (51.74±5.2*) and HDL (29.5±4.1*) levels (mg/dl) leads to hypercholesterolaemia when compared with normal rats. Moreover, the serum concentration of HDL was significantly reduced in rats fed with high fat diet for 28 days (Table 1).

Table 1: Effect of high fat diet on serum lipid profile

S.No.	Cholesterol	Normal Control	High Fat Diet Treated Rats (Hypercholesterolaemic Rats)
1.	**Total Cholesterol**	98.22±8.2	272.1±24.4*
2.	**Triglycerides**	107.25±8.9	258.7±19.7*
3.	**LDL**	28.97±3.2	190.86±18.8*
4.	**VLDL**	21.45±2.6	51.74±5.2*
5.	**HDL**	47.8±4.3	29.5±4.1*

**P<0.05 vs Control*

The lipid peroxidation measured in terms of increased TBARS and superoxide anion generation with consequent decrease in GSH were noted in hypercholesterolaemic rat hearts subjected to 30 min of global ischemia and 120 min of reperfusion, as compared to normal (Table 3-5). Moreover, hypercholesterolaemic rat hearts showed high oxidative stress when compared with normal rat hearts subjected to I/R (Table 3-5). Four episodes of IPC markedly attenuated the I/R-induced oxidative stress in normal rat hearts as assessed in terms of reduction in TBARS and superoxide anion generation and consequent increase in reduced GSH. However, IPC mediated reduction in oxidative stress against I/R was markedly abolished in hypercholesterolaemic rat hearts (Table 3-5).

Global ischemia followed by reperfusion significantly increased LDH and CK-MB levels in coronary effluent in normal and hypercholesterolaemic rat hearts (Table 7-8). Maximum release of LDH was noted immediately after reperfusion, whereas peak release of CK-MB was noted at 5 min of reperfusion. Further, I/R were noted to increase the infarct size in normal and hypercholesterolaemic rat hearts (Table 6). Moreover, hypercholesterolaemic rat hearts showed enhanced myocardial injury when compared with normal rat hearts subjected to I/R. The IPC afforded cardioprotection in normal rat hearts by significantly attenuating I/R-induced myocardial injury as assessed in terms of reduction in LDH and CK-MB levels and myocardial infarct size (Table 7-8). However, the IPC mediated cardioprotection against I/R-injury was markedly abolished in hypercholesterolaemic rat hearts. Global ischemia followed by reperfusion significantly decreased the amount of coronary perfuaste in normal and hypercholesterolaemic rat hearts (Table 2). In addition, the hypercholesterolaemic rat hearts showed marked reduction in coronary perfusate as compared to normal rat hearts (Table 2). The IPC significantly improved the coronary flow rate in normal rat hearts. On the other hand, the IPC has failed to improve the coronary flow rate in hypercholesterolaemic rat hearts subjected to I/R (Table 2).

Effect of Clofibrate in I/R-induced Oxidative Stress and Myocardial Injury in normal and hypercholesterolaemic rat hearts

Hypercholesterolaemic rat hearts showed high degree of oxidative stress and enhanced myocardial injury as compared with normal rat hearts subjected to I/R. Treatment with Clofibrate (300mg/kg/day, *i.p.*, for 2 weeks) markedly reduced the oxidative stress produced as a result of I/R in normal rat hearts as assessed in terms of reduction in TBARS and superoxide anion generation and consequent increase in reduced form of GSH; but its treatment partially reduced the I/R-induced oxidative stress in hypercholesterolaemic rat hearts (Table 3-5). Treatment with Clofibrate (300mg/kg/day, *i.p.*, for 2 weeks) markedly reduced the I/R-induced myocardial injury in normal rat hearts as assessed in terms of reduction in myocardial infarct size, decrease in LDH and CK-MB levels and improvement in coronary flow rate (Table 2). On the other hand, Clofibrate treatment partially reduced I/R-induced myocardial injury in hypercholesterolaemic rat hearts (Table 6-8).

Effect of Clofibrate IPC-Mediated Myocardial Protection in Normal Rat Hearts

Pretreatment with Clofibrate (300mg/kg/day, *i.p.*, for 2 weeks) has not affected the IPC-induced attenuation I/R-mediated oxidative stress in normal rat hearts. Moreover, its pretreatment has not modulated the IPC-induced reduction in infarct size, LDH and CK-MB levels and improvement in coronary flow rate in normal rat hearts subjected to I/R (Table 3-8).

Effect of Clofibrate in Abrogated Cardioprotective Potential of IPC in Hypercholesterolaemic Rat Hearts

Treatment with Clofibrate (300mg/kg/day, *i.p.*, for 2 weeks) did not affect the cardioprotective effects of IPC in normal rat hearts subjected to I/R. On the other hand, its pretreatment markedly restored the cardioprotective potential of IPC in hypercholesterolaemic rat hearts subjected to I/R as assessed in terms of improvement in coronary flow rate and reduction in myocardial infarct size, LDH, CK-MB and oxidative stress (Table 3-8).

DISCUSSION

Increase in infarct size and the release of LDH and CK-MB are documented to be an index of I/R-induced myocardial injury [31]. In the present study, 30 min of ischemia followed by 120 min of reperfusion was noted to produce myocardial injury as assessed in terms of increased infarct size in the heart and elevated release of LDH and CK-MB in coronary effluent, which were consistent with earlier reports [32]. The maximal release of LDH was noted immediately after reperfusion whereas the peak release of CK-MB was observed after 5 min of reperfusion, which are in accordance with earlier studies. Also, the increase in lipid peroxidation and superoxide anion generation with consequent decrease in the reduced glutathione levels have been suggested to be the indicators of oxidative stress [33-34]. This suggests the development of I/R-induced oxidative stress, which may be responsible for the noted I/R-induced myocardial injury in the present study. In the present study, a significant decrease in coronary flow rate and marked increase in infarct size, release of LDH and CK-MB were noted in hypercholesterolaemic rat hearts as compared with the normal rat hearts subjected to I/R.

High fat diet for 28 days significantly increased serum concentrations of total cholesterol, triglycerides, LDL and VLDL. Moreover, the serum concentration of HDL was significantly reduced in rats fed with high fat diet for 28 days. Hcl has been noted to modulate the severity of I/R-induced myocardial injury and interfere with the cardioprotective potential of IPC [35]. Moreover, Hcl possesses an important risk factor for coronary heart disease.

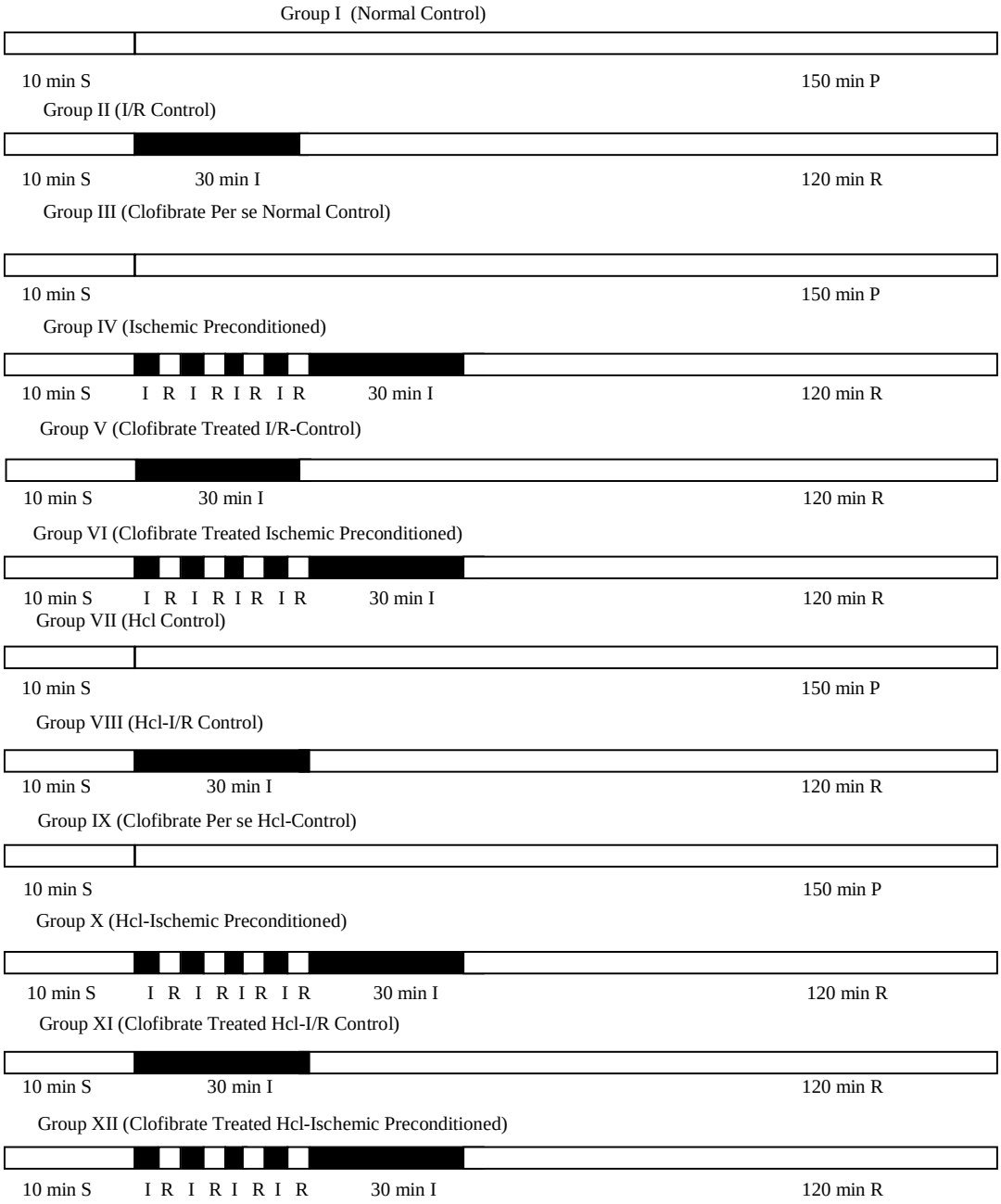

Figure 1: Diagrammatic representation of experimental protocol

S indicates stabilization; I indicate global ischemia; R indicates reperfusion with K-H solution; I/R indicates ischemia-reperfusion injury; Ischemic preconditioned indicates ischemic preconditioned normal rat heart; Hcl indicates hypercholesterolaemia

Hcl has been reported to decrease myocardial NO concentration, causes generation of ROS like superoxide anion and peroxynitrite radical, activates apoptotic caspase-3 and lead to accumulation of cholesterol in the sarcolemmal and mitochondrial membranes [13,35] that may attenuate the cardioprotective effect of IPC in hypercholesterolaemic states. Thus, the observed marked increase in myocardial injury in hypercholesterolaemic rat hearts may be due to the development of high degree of oxidative stress. This contention is supported by the fact that

a marked increase in lipid peroxidation and superoxide anion generation and subsequent decrease in glutathione level were noted in hypercholesterolaemic rat hearts when compared with normal rat hearts subjected to I/R.

Table 2: Effect of Clofibrate and IPC on Coronary Flow Rate (CFR) (ml/min)

Groups	Basal	0 min	5 min	30 min	120 min
Normal Control	6.9 ± 0.76	7.1 ± 0.77	6.9 ± 0.72	6.7 ± 0.73	6.5 ± 0.69
I/R Control	7.1 ± 0.73	2.6 ± 0.31	4.4 ± 0.54	3.6 ± 0.31	2.9 ± 0.32a
Clo Per se Normal Control	7.2 ± 0.68	7.2 ± 0.69	7.5 ± 0.81	7.2 ± 0.79	6.8 ± 0 .77
IPC Control	7.2 ± 0.79	4.8 ± 0.54	5.4 ± 0.58	5.1 ± 0.58	4.9 ± 0.45b
Clo Treated I/R Control	7.4 ± 0.77	4.3 ± 0.41	5.1 ± 0.55	4.8 ± 0.45	4.4 ± 0.57b
Clo Treated IPC	6.9 ± 0.72	4.6 ± 0.52	5.5 ± 0.63	5.1 ± 0.56	4.9 ± 0.55b
Hcl-Control	7.2 ± 0.81	7.1 ± 0.79	7.2 ± 0.82	7.0 ± 0.78	6.9 ± 0.79
Hcl-I/R Control	7.2 ± 0.76	2.9 ± 0.33	3.7 ± 0.42	3.4 ± 0.44	2.1 ± 0.29c,d
Clo Per se Hcl-Control	7.1 ± 0.78	7.2 ± 0.69	7.0 ± 0.78	7.4 ± 0.82	7.0 ± 0.69
Hcl-IPC Control	7.3 ± 0.81	3.1 ± 0.49	3.5 ± 0.41	2.8 ± 0.25	2.3 ± 0.32
Clo Treated Hcl-I/R Control	7.4 ± 0.79	4.0 ± 0.47	4.2 ± 0.52	3.5 ± 0.42	2.8 ± 0.39e
Clo Treated Hcl-IPC	6.9 ± 0.72	4.2 ± 0.39	5.1 ± 0.55	4.8 ± 0.57	4.7 ± 0.52e,f

Values are expressed as mean ±S.D. a = p < 0.05 vs Normal Control; b = p < 0.05 vs I/R Control; c = p < 0.05 vs Hcl-Control; d = p < 0.05 vs I/R Control; e = p < 0.05 vs Hcl-IR Control; f = p < 0.05 vs Hcl-Ischemic Preconditioned

Table 3: Effect of Clofibrate and IPC in I/R-induced increase in TBARS level

Groups	TBARS (nM/gm wet tissue weight)
Normal Control	30.4 ± 3.5
I/R Control	76.7 ± 5.7[a]
Clo Per se Normal Control	35.3 ± 3.8
IPC Control	52.3 ± 4.5[b]
Clo Treated I/R Control	55.6 ± 4.1[b]
Clo Treated IPC	54.3 ± 5.2[b]
Hcl-Control	38.7 ± 3.3
Hcl-I/R Control	99.8 ± 6.8[c,d]
Clo Per se Hcl-Control	45.2 ± 4.1
Hcl- IPC Control	93.4 ± 6.3
Clo Treated Hcl-I/R Control	80.7 ± 4.4[e]
Clo Treated Hcl-IPC	67.8 ± 4.1[e,f]

Values are expressed as mean ±S.D. a = p< 0.05 vs Normal Control; b = p< 0.05 vs I/R Control; c = p< 0.05 vs Hcl-Control; d = p< 0.05 vs I/R Control; e = p< 0.05 vs Hcl-IR Control; f = p< 0.05 vs Hcl-Ischemic Preconditioned (IPC)

Table 4: Effect of Clofibrate and IPC in I/R-induced increase in superoxide anion level (expressed as reduced NBT)

Groups	Reduced NBT (pM/min/mg wet tissue weight)
Normal Control	19.8 ± 2.2
I/R Control	65.4 ± 5[a]
Clo Per se Normal Control	23.1 ± 2.2
IPC Control	40.2 ± 3.8[b]
Clo Treated I/R Control	38.6 ± 3.4[b]
Clo Treated IPC	41.2 ± 4.2[b]
Hcl-Control	22.1 ± 2
Hcl-I/R Control	87.5 ± 5[c,d]
Clo Per se Hcl-Control	21.1 ± 2.1
Hcl- IPC Control	78.1 ± 5.1
Clo Treated Hcl-I/R Control	64.5 ± 4.5[e]
Clo Treated Hcl-IPC	47.6 ± 3.2[e,f]

Values are expressed as mean ±S.D. a = p< 0.05 vs Normal Control; b = p< 0.05 vs I/R Control; c = p< 0.05 vs Hcl-Control; d = p< 0.05 vs I/R Control; e = p< 0.05 vs Hcl-IR Control; f = p< 0.05 vs Hcl-Ischemic Preconditioned (IPC)

IPC has been well documented to produce myocardial protection against I/R-induced myocardial injury[3,7]. The mechanisms involved in the cardioprotective potentials of IPC are activation of PI3K/Akt and eNOS, release of NO, closure of MPTP, opening of K_{ATP}-channels and reduction in reperfusion-induced oxidative stress [36-37]. In the present study, IPC was noted to reduce I/R-induced myocardial injury in normal rat hearts as assessed in terms of reductions in infarct size, release of LDH and CK-MB and oxidative stress. However, the cardioprotective effect of IPC was insignificant in Hcl rat hearts with high degree of noted oxidative stress. Thus, it is strongly suggested that

the high degree of oxidative stress developed in Hcl rat hearts may be responsible for the observed paradoxical effect of IPC.

Table 5: Effect of Clofibrate and IPC in I/R-induced decrease in reduced GSH level

Groups	GSH (uM/mg wet tissue weight)
Normal Control	0.763 ± 0.033
I/R Control	0.598 ± 0.041[a]
Clo Per se Normal Control	0.782 ± 0.038
IPC Control	0.892 ± 0.045[b]
Clo Treated I/R Control	0.914 ± 0.038[b]
Clo Treated IPC	0.929 ± 0.033[b]
Hcl-Control	0.797 ± 0.051
Hcl-I/R Control	0.473 ± 0.049[c,d]
Clo Per se Hcl-Control	0.753 ± 0.041
Hcl- IPC Control	0.539 ± 0.046
Clo Treated Hcl-I/R Control	0.685 ± 0.037[e]
Clo Treated Hcl-IPC	0.806 ± 0.044[e,f]

Values are expressed as mean ±S.D. a = p< 0.05 vs Normal Control; b = p< 0.05 vs I/R Control; c = p< 0.05 vs Hcl-Control; d = p< 0.05 vs I/R Control; e = p< 0.05 vs Hcl-IR Control; f = p< 0.05 vs Hcl-Ischemic Preconditioned (IPC)

Table 6: Effect of Clofibrate and IPC in I/R-induced increase in infarct size

Groups	% Infarct Size
Normal Control	8 ± 1.2
I/R Control	47.5 ± 3.1[a]
Clo Per se Normal Control	8.2 ± 1.6
IPC Control	23.4 ± 2.6[b]
Clo Treated I/R Control	25.2 ± 2.5[b]
Clo Treated IPC	23.2 ± 1.9[b]
Hcl-Control	8.8 ± 1.6
Hcl-I/R Control	59.2 ± 4.8[c,d]
Clo Per se Hcl-Control	8.2 ± 1.9
Hcl-IPC Control	51.2 ± 4.5
Clo Treated Hcl-I/R Control	42.4 ± 3.3[e]
Clo Treated Hcl-IPC	33.2 ± 3.4[e,f]

Values are expressed as mean ±S.D. a = p< 0.05 vs Normal Control; b = p< 0.05 vs I/R Control; c = p< 0.05 vs Hcl-Control; d = p< 0.05 vs I/R Control; e = p< 0.05 vs Hcl-IR Control; f = p< 0.05 vs Hcl-Ischemic Preconditioned (IPC)

Table 7: Effect of Clofibrate and IPC in I/R-induced increase in CK-MB level

Groups	CK-MB (U/L)
Normal Control	33.2 ± 5.8
I/R Control	166.5 ± 11.1[a]
Clo Per se Normal Control	35.4 ± 4.9
IPC Control	71.5 ± 9[b]
Clo Treated I/R Control	82.3 ± 7.9[b]
Clo Treated IPC	76.5 ± 6.9[b]
Hcl-Control	29.9 ± 5.6
Hcl-I/R Control	199.2 ± 15.1[c,d]
Clo Per se Hcl-Control	36.2 ± 3.8
Hcl-IPC Control	177.6 ± 14.8
Clo Treated Hcl-I/R Control	142.3 ± 9.9[e]
Clo Treated Hcl-IPC	115.4 ± 7.1[e,f]

Values are expressed as mean ±S.D. a = p< 0.05 vs Normal Control; b = p< 0.05 vs I/R Control; c = p< 0.05 vs Hcl-Control; d = p< 0.05 vs I/R Control; e = p< 0.05 vs Hcl-IR Control; f = p< 0.05 vs Hcl-Ischemic Preconditioned (IPC)

Pretreatment with Clofibrate (300mg/kg/day, *i.p.*, for 2 weeks) did not affect the cardioprotective effect of IPC in normal rat hearts; but its pretreatment significantly restored the cardioprotective effect of IPC in Hcl rat hearts. Clofibrate has been well reported to be a selective synthetic agonist of PPAR-α [23,38]. Thus, it is suggested that activation of PPAR-α in ischemic myocardium may play a pivotal role in the attenuation of cardioprotective potential of IPC in Hcl rat hearts. The signaling mechanisms such as activation of PI3K/Akt, subsequent activation of eNOS and generation of NO have been well implicated in IPC mediated cardioprotection. It has been well reported that Hcl down regulates eNOS and reduces the generation and bioavailability of NO [21]. Moreover,

various experimental studies have reported that Hcl increase oxidative stress significantly [39]. Further, activation of PPAR-α has been reported to activate PI3K/Akt pathway [22]. Since, Clofibrate has restorted the cardioprotective effect of IPC in Hcl rat hearts, it may be suggested that PPAR-α mediated activation of PI3K/Akt-eNOS pathway in hypercholesterolaemic rat hearts may be responsible for the restoration of cardioprotective potential of IPC.

Table 8: Effect of Clofibrate and IPC in I/R-induced increase in LDH level

Groups	LDH (U/L)
Normal Control	35.8 ± 5.3
I/R Control	255.1 ± 14.9[a]
Clo Per se Normal Control	39.8 ± 4.1
IPC Control	178.6 ± 12.8[b]
Clo Treated I/R Control	185.4 ± 11.2[b]
Clo Treated IPC	188.5 ± 14.1[b]
Hcl-Control	40.3 ± 6.1
Hcl-I/R Control	292.1 ± 18.9[c,d]
Clo Per se Hcl-Control	48.9 ± 5.1
Hcl-IPC Control	267.7 ± 18.5
Clo Treated Hcl-I/R Control	242.1 ± 14.2[e]
Clo Treated Hcl-IPC	208.5 ± 11.3[e,f]

Values are expressed as mean ±S.D. a = p< 0.05 vs Normal Control; b = p< 0.05 vs I/R Control; c = p< 0.05 vs Hcl-Control; d = p< 0.05 vs I/R Control; e = p< 0.05 vs Hcl-IR Control; f = p< 0.05 vs Hcl-Ischemic Preconditioned (IPC)

In addition, PPAR-α activation has been noted to diminish ROS generation and postischemic cardiomyocytic apoptosis [22,40]. Reperfusion-induced ROS production has been noted to down regulate PPAR-α expression which is detrimental for maintaining contractile function of the heart. Thus, it may be suggested that down regulation of PPAR-α by ROS may be associated with cardiac dysfunction in Hcl rat hearts subjected to I/R. Moreover, activation of PPAR-α has been shown to decrease the expression of pro-inflammatory cytokines and involve in oxidative stress-induced apoptotic cell death [38, 41-42]. Thus, it could be suggested that PPAR-α activation during reperfusion may be responsible for the decrease in generation of high amount of ROS in Hcl rat hearts possibly by involving the well established IPC-mediated cardioprotective PI3K/Akt/eNOS pathway. This contention is supported by the results obtained in the present study that pretreatment with Clofibrate has restorted the cardioprotective and infarct size limiting properties of IPC in Hcl rat hearts as assessed in terms of reductions of CK-MB and LDH in coronary effluent along with decreased oxidative stress in Hcl rat hearts. Our study for the first time reports that Clofibrate has significant role in the restoration of abrogated cardioprtective effect of IPC in hypercholesterolaemic rat hearts. Hence, it can be postulated that the selective PPAR-α agonists may be the potential candidates for providing pharmacological preconditioning in hypercholesterolaemic patients in order to afford cardioprotection. However, further studies measuring the PPAR-α expression during hypercholesterolaemic condition may be warranted.

CONCLUSION

On the basis of above discussion, it may be concluded that there may be down regulation of PPAR-α signaling during hypercholesterolaemic condition that consequently produced high degree of oxidative stress, which may be responsible to abolish the cardioprotective potential of IPC against I/R induced myocardial injury in hypercholesterolaemic rat hearts. The PPAR-α activation by Clofibrate restored the attenuated cardioprotective effect of IPC in hypercholesterolaemic rat hearts.

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