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Reciprocalcation of caveolin and HSP-72 on IPC interceded cardio-protection in the orchidectomized rats

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ABSTRACT

Diminished testosterone levels conjoined to cardiovascular risk factor mainly myocardial infarction which broadens the risk of cardiovascular mortality referring to age. Ischemic preconditioning (IPC) is one of the interventions to shield such injury. The present study implicated the possible involvement of caveolin and heat shock protein 72 (HSP-72) during stress in orchidectomy (OCD) challenged rats. OCD was performed in male rats and kept for 6 weeks to observe the reduction in the level of testosterone. Isolated perfused heart of normal and OCD group was subjected to ischemic insult as per IPC cycle. Myocardial infarct size, haemodynamic, enzymatic and oxidative stress parameter were assessed for each heart. Diadzein (DDZ) a caveolin inhibitor was administered before the isolation of heart and it significantly decreases myocardial infarct size, release of lactate dehydrogenase, creatinine kinase and oxidative stress marker. DDZ also potentiated the effect IPC-mediated increase in the heart rate and coronary flow. The effect of caveolin inhibitor was remarkably reduced by quercetin administered before 1 h. of the administration DDZ. The findings of this study revealed that protection of myocardium induced by caveolin inhibitor pretreatment has not been lost in OCD rat heart.

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INTRODUCTION

In the current scenario, a significant reason of mortality and morbidity is cardiac disease (Murray and Lopez, 1997). The lower level of testosterone has been associated with all cause of mortality in cardiovascular risk. The normal functioning of myocardium is restored by reperfusion of an ischemic myocardium (Chrisostome, 2012). Ischemic preconditioning (IPC) is an inconceivable endogenic defensive mechanism in which short unpredictable episodes of sublethal ischemia followed by reperfusion before the subsequent postponed ischemic attack improves the obstruction against stress (Muller and Dhalla, 2010). The cardio-protection is regulated by various signaling pathways including opening of mitochondrial K_{ATP} channel, phosphorylation of eNOS, nitric oxide generation and AKT/PI3K (Sharma *et al.*, 2010). The myocardium protective effect of IPC reduced in certain morbid situations like diabetes mellitus (Ajmani *et al.*, 2011; Yadav, 2010), hyperlipidemia (Yadav *et al.*, 2010), hyperten-

sion (Snoeckx *et al.*, 1986, 1993), heart failure (Ferdinandy *et al.*, 1998, 2007) and aging (Abete *et al.*, 1996).

The heat shock protein (HSP-72) synthesis induced by stress response and molecular chaperone plays a major part in healing of cell from stress and defend the myocardium cell from successive insults. The molecular chaperone of HSP-72 and their co-proteins linked with signaling molecules (Asea, 2005). HSP-72 has ability to protect the stress cell from concede incipient polypeptides, unbalanced portions of amino acids and its sequences. Moreover, the molecular chaperone has creased the stress proteins and averts their accumulation with other proteins (Chow *et al.*, 2009). The various intrusions like opioids, bradykinin, adenosine, norepinephrine, IPC and HSP-72 have been accounted for to lower the myocardial infarct size, when given 24 h before of ischemia (Fryer, 2002). Also, recommended that decrease in myocardial content of HSP-72 may decrease the ischemic resistance (Guisasola *et al.*, 2006).

Caveolae are lipid raft located at plasma membrane consist of cytoskeleton protein with several subunits (Caveolin 1-3). Caveolins are vital basic segments of caveolae, caveolin proteins capacity to take up the lipids and proteins to caveolae for cooperation in molecular signaling of cell segments and activity in cell signaling (Goyal *et al.*, 2016). It has been reported that IPC are effective against stress prompted injury by phosphorylation and inhibition of caveolin (Schilling *et al.*, 2018). But in diseased condition IPC cycle was failed. So the current investigation has been designed to evaluate the significance of caveolin and HSP-72 interaction in the regulation of cardio-protective impact, in OCD challenged rats.

MATERIALS AND METHODS

Animals

Wistar rats (male) weigh about 180-210 gm were used and kept in the animal cages following a cycle of 12-h light/12-h dark. All the experimental work performed by following the national guideline of laboratory animals and the protocol was approved by Institutional Animal Ethics Committee (KNIMT/PHAR/IAEC/18/01).

Chemicals and Drugs

Diadzein (DDZ) (Sigma Aldrich Pvt. Ltd, India), Quercetin (Helix Bioscience, India) was procured. The Krebs-Henseleit (KH) buffer solution and all the reagents were freshly prepared for the experiment before use.

Procedure of orchidectomy

For removal of testis, sprit was used to clean the scrotal sac and a small incision of about 2cm was made mid sagitally at the scrotal septum for producing orchidectomy. Carefully dissected the spermatic cord, tied and cut then removed the both testes from the scrotal sac. The incision was sutured using sterilized items. Antibiotic powder was applied to wounds and allowed to recover (Sadri and Ahmadi, 2013).

Isolated rat heart preparation

The rats were anesthetized by an intramuscular injection of sodium pentobarbital (60 mg/kg) and the isolated rat heart was stored in heparinized KH solution ($MgSO_4.7H_2O$ 1.2 mM; $CaCl_2$ 2.5 mM; KCl 4.7 mM; NaCl 118 mM; glucose- 11m M; $NaHCO_3$ 25 mM; KH_2PO4 1.2 mM, to get pH 7.4). Excised hearts were immediately hanged on Langendorff's apparatus for further experimentation. The isolated heart preparation was perfused with KH buffer solution while maintaining temperature to 37°C, and passing bubble of 5% CO_2 and 95% O_2 (Hosseini *et al.*, 2020). At the end of stabilization phase, 0, 30, 120 min after ischemia, the coronary fluid was collected for estimation of LDH, CK-MB, coronary flow and cardiac electrogram was also monitored for heart rate.

Experimental protocol

The experiment was conducted on five groups of male Wistar rats and each group contained six rats (n=6). The detailed groups of experiment shown in Figure 1 and described here:

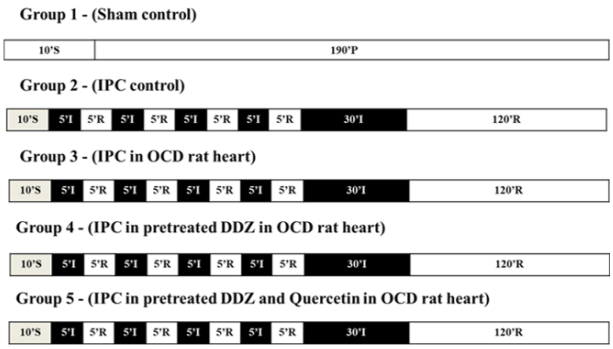

Figure 1: Detailed procedures of experimentation

Sham Control, (n = 6): Isolated normal rat heart was subjected to 10 min of stabilization and then perfused with KH buffer for 190 min continuously. At this stage, there was no global ischemia.

IPC Control, (n=6): Isolated normal rat heart was kept for 4 short episode of IPC after 10 min of stabilization. Each short episode of IPC consists of 5 min global ischemia following reperfusion of 5 min with

KH buffer solution which was further continued to global ischemia of 30 min and 120 min reperfusion.

IPC in OCD rat, (n= 6): Isolated OCD rat heart was kept for 4 short episode of IPC as reported earlier in group-2.

IPC in pretreated with DDZ in OCD rat heart, (n= 6): Isolated pretreated OCD rat heart with DDZ (0.2 mg/kg/s.c/day dose was given for a Week) was kept for 4 short episode of IPC and rest protocol as described in group-2.

IPC in pretreated with DDZ and Quercetin in OCD rat, (n= 6): Isolated pretreated OCD rat heart with DDZ (0.2 mg/Kg/s.c/day dose was given for a week) and at before 24 h of isolation of heart quercetin (4 mg/kg, i.p.) was given 1 h before subcutaneous application of DDZ in OCD rats, rest protocol as described in group-2.

Measurement of infarct size

After the completion of IPC-mediated cycle the isolated heart was stored at −80°C for 20 to 30 min. Transverse slices were obtained after cutting the frozen heart from apex to base. Each slide was measured with a thickness of 2 to 3 mm. The TTC (triphenyl-tetrazolium chloride) solution was used to stain prepared slices. The brick red color was stained for living myocardial tissues, while, infarct area remained unstained. The % of infarct area was measured using Image J-software in about total area of heart (NIH, Bethesda, MD, USA) (Varshney *et al.*, 2017).

Measurement of cellular injury

The coronary effluent from heart preparation, LDH and CKMB levels were determined to assess the range of myocardium injury in experimental rats. At the end of investigation the collected samples have been estimated by spectrophotometrically in the perfusate using commercial detection kits (Coral Clinical Systems Pvt. Ltd., India) (Charan *et al.*, 2016).

Measurement of oxidative stress marker

Heart tissue samples were softened at 4°C and the homogenate was prepared by using a homogenizer at 6000 rpm for 5 min in 0.1 M phosphate buffer (pH 7.4, 10% w/v). Which was again centrifuged at 3000 rpm for 10 min and 2 ml of supernatant was separated. Proteins were separated from remaining tissue homogenate by adding an equal volume of 5% trichloroacetic acid (TCA) then supernatant was stored by separating at 4000 rpm for 10 min.

Superoxide dismutase activity measurement

This activity in the heart was determined by spectrophotometrically at 560 nm. Prepare a layout for 96 plates of blank, standard and tissue samples. In blank, added 300μl of tris buffer, in autoxidation added 290μl of tris buffer and in well of all samples, first we added 10μl tissue homogenate then 280μl of tris buffer and finally added 10μl of pyrogallol by multichannel pipette in each well except blank (Marklund and Marklund, 1974).

Catalase activity measurement

Break down of H_2O_2 in heart ocurred the presence of catalase (CAT); such changes were measured using a spectrophotometer at 240 nm. Results of this activity are expressed in terms of CAT activity/min/mg of the protein (Pachauri *et al.*, 2017).

Glutathione activity measurement

This activity estimation was based on GPX catalyzed oxidation of glutathione by the action of cumene hydroperoxide. Procedure estimates as reduced glutathione level were determined using the method described by Ellman (1959).

Statistical analysis

All data analysis was done by software Graph Pad Prism version 7. The statistical values were indicated as mean±SEM. Newman-keul post hoc test was applied for all statistical data followed by one way ANOVA analysis. Statistical significant values were considered as p-value of less than 0.05.

RESULTS

Role of orchidectom y on testosterone level

The orchidectomy significantly decreases the level of testosterone as compared to sham control group. But from the other area also produces less amount of testosterone so the level did not become zero (Figure 2).

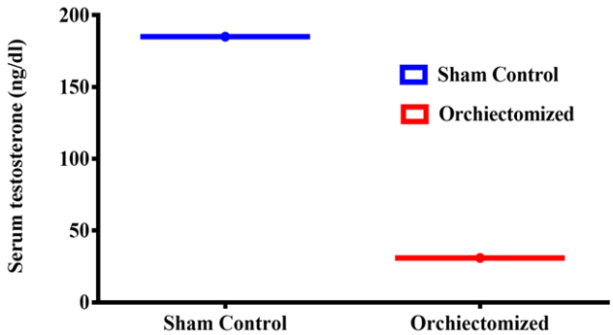

Figure 2: Effect of orchidectomy on testosterone level. All values are mean+SEM

Role of DDZ on coronary flow

During investigation it was noted that at basal time no remarkable alterations found in all sets of experiment. But on 0 and 30 min of IPC mediated OCD

Table 1: DDZ restored in IPC-induced alteration in coronary flow in OCD rat heart

Parameter	Sham Control	IPC Control	IPC+OCD	IPC+OCD+ DDZ	IPC+OCD+DDZ+ Quercetin
			Coronary flow (mL/min)		
Basal	13.6±0.4	11.3±0.3	11.2±0.3	11.8±0.4	12.2±0.42
0 min	12.5±0.51	9.1±0.39	7.8±0.35$^{\#}$	9.20±0.4$^{\#\#}$	8.79±0.41$^{\#\#\#}$
30min	10.9±0.4	7.9±0.38	7.1±0.35$^{\#}$	7.55±0.4$^{\#\#}$	7.42±0.32$^{\#\#\#}$

All value are mean±SEM.$^{\#}$P<0.05 vs. IPC group, $^{\#\#}$P<0.05 vs. IPC+OCD group, $^{\#\#\#}$P<0.05 vs. IPC+OCD+DDZ group. IPC= Ischemic preconditioning, OCD= Orchidectomy, DDZ= Daidzein.

Table 2: DDZ restored in IPC-induced alteration in heart rate in OCD rat heart

Parameter	Sham Control	IPC Control	IPC+OCD	IPC+OCD+ DDZ	IPC+OCD+DDZ+ Quercetin
			Heart rate (Beats/min)		
Basal	388±15	384±17	380±24	378±15	382±12
0 min	384±12	345±10	331±16$^{\#}$	372±12$^{\#\#}$	348±16$^{\#\#\#}$
30min	383±14	314±15	301±16$^{\#}$	381±13$^{\#\#}$	341±18$^{\#\#\#}$

All value are mean±SEM.$^{\#}$P<0.05 vs. IPC group, $^{\#\#}$P<0.05 vs. IPC+OCD group, $^{\#\#\#}$P<0.05 vs. IPC+OCD+DDZ group. IPC= Ischemic preconditioning, OCD= Orchidectomy, DDZ= Daidzein.

Table 3: DDZ restored in IPC-induced alteration in oxidative marker in OCD rat heart

Oxidative stress markers	GSH level (units/ min/mg protein)	SOD level (units/ min/mg protein)	CAT level (units/ min/mg protein)
Sham control	3.39±0.081	0.71±0.048	3.92±0.24
IPC control	2.13±0.080	0.63±0.02	3.49±0.18
IPC+OCD	0.98 ±0.072$^{\#}$	0.51 ±0.03$^{\#}$	2.5±0.15$^{\#}$
IPC+OCD+DDZ	1.6 ±0.084$^{\#\#}$	0.53±0.011$^{\#\#}$	2.9±0.16$^{\#\#}$
IPC+OCD+DDZ-+Quercetin	0.98±0.06$^{\#\#\#}$	0.51±0.06$^{\#\#\#}$	2.1±0.18$^{\#\#\#}$

All value are mean±SEM. $^{\#}$P<0.05 vs. IPC group, $^{\#\#}$P<0.05 vs. IPC+OCD group, $^{\#\#\#}$P<0.05 vs. IPC+OCD+DDZ group. IPC= Ischemic preconditioning, OCD= Orchidectomy, DDZ= Daidzein.

induced groups showed decrease in coronary flow. In OCD rats heart DDZ potentiate the IPC interceded rise in coronary flow. In addition quercetin along with DDZ didn't make any remarkable alteration to IPC interceded rise in coronary flow (Table 1).

Role of DDZ on heart rate

During investigation it was found that at basal time there were no significant alterations found in heart rate in all groups of experiment. But at 0 and 30 min of IPC mediated OCD induced decrease in heart rate. In OCD rat heart DDZ potentiate IPC interceded rise in heart rate. In addition quercetin along with DDZ didn't make any remarkable alteration to IPC interceded rise in heart rate (Table 2).

Role of DDZ on infarct size

The short episodes of IPC cycle were remarkable increase them myocardial infarct size in OCD rats.

Pre-treatment of DDZ remarkable reestablish IPC-interceded lessening in cardiac injury in OCD rats heart, and quercetin remarkable weakened the decrease of infarct size in OCD rat heart (Figure 3). All value are mean±SEM.$^{\#}$P<0.05 vs. IPC group, $^{\#\#}$P<0.05 vs. IPC+OCD group, $^{\#\#\#}$P<0.05 vs.

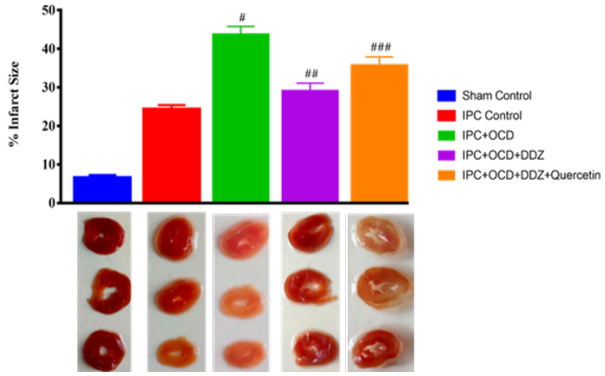

Figure 3: DDZ restored in IPC-mediated alteration in infarct size in OCD rats heart

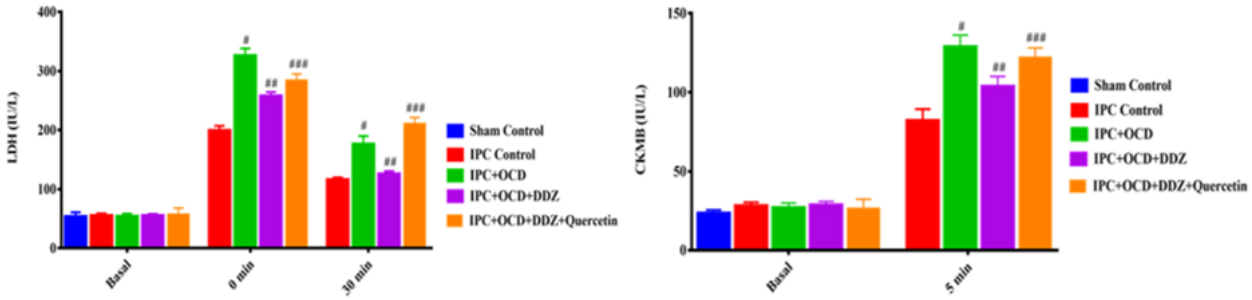

Figure 4: DDZ restored in IPC-induced alteration in LDH and CKMB in OCD rat heart

IPC+OCD+DDZ group. IPC= Ischemic preconditioning, OCD= Orchidectomy, DDZ= Daidzein.

Role of DDZon CK-MB and LDH

At basal time no remarkable changes found in CK-MB and LDH activity in rat coronary fluid in all sets of experiment. But on 0 min, IPC diminish the OCD-prompt rise in the level of LDH and at 5 min IPC diminish OCD-prompted rise the CK-MB level in coronary fluid. DDZ decrease the LDH and CK-MB level in coronary fluid in OCD challenged rats. But, DDZ with quercetin increases the LDH and CK-MB level in coronary fluid in OCD challenged rats (Figure 4). All value are mean±SEM. $^{\#}P<0.05$ vs. IPC group, $^{\#\#}P<0.05$ vs. IPC+OCD group, $^{\#\#\#}P<0.05$ vs. IPC+OCD+DDZ group. IPC= Ischemic preconditioning, OCD= Orchidectomy, DDZ= Daidzein.

Role of DDZ on oxidative stress

The levels of GSH, SOD and CAT were significantly reduced in OCD rat heart in cardiac tissue. But during IPC cycle in normal rat heart it increase the level of GSH, SOD and CAT. DDZ enhanced the IPC-mediated rise in levels of SOD, CAT and GSH in OCD rat heart. But, DDZ with quercetin decrease the level of oxidative marker in OCD rat heart (Table 3).

DISCUSSION

The current investigation shows that DDZ enhances the protection of myocardium through IPC in OCD challenged rat. DDZ improved the IPC-mediated reduction in myocardial infarct size, haemodynamic, enzymatic and oxidative stress in OCD challenged rats. In addition quercetin (HSP-72 inhibitor) repealed the increasing effect of DDZ on IPC-mediated cardio-protection in OCD challenged rat. Hence, it may be expected that DDZ and HSP-72 could increases the effect of IPC through caveolin mediated mechanism pathway during testosterone deficiency induced cardiac injury.

The new insight of current investigation is that the pretreatment of DDZ increases the IPC-mediated coronary flow and heart rate. However, the increases effect of DDZ was not observed with quercetin. The finding revealed that the effect of DDZ on IPC-induced cardiac protection may involve caveolin inactivation and HSP-72 activation.

Cardiac damage was noticed as myocardial infarct size increases with release of LDH and CK-MB. CK-MB and LDH are pathological markers. In this investigation, four short events of ischemia and reperfusion in OCD rats increase the myocardial infarct size and releases of CKMB and LDH. Moreover, when we given pretreatment of DDZ with four cycles of ischemia and reperfusion (5 min each) and preceding with 30 min of ischemia and 120 min of reperfusion were significantly decreases the infarct size and release of CK-MB, LDH in OCD challenged rat heart.

This shows the IPC mediated cardio-protection by inhibition of caveolin is mediated through HSP-70. Caveolae are plasma membrane invaginations (50-100 nm) located on the surface of endothelial cells with caveolin proteins and it act as a signaling platform for various receptor and molecules (Goyal *et al.*, 2016). IPC can modify the regulatory mechanism of caveolin and stimulate the molecular signaling require in the protection of heart against stress (Horikawa *et al.*, 2014). It is also documented that expression of caveolin is unregulated in testosterone deficiency rat heart (Oh *et al.*, 2011), and the IPC cycle also failed during pathological condition (Ajmani *et al.*, 2011). The shorter episodes of ischemia have activated generation of HSP-72 in the heart, it's enhances the resistance against cardiac injury (Fryer, 2002). DDZ can decrease the caveolin expression and facilitate the IPC (Sharma *et al.*, 2012; Sobey *et al.*, 2004). HSP-72 protein has the property of protecting enzymes and conformational changes in reactive oxygen species. It is also reported that the role of HSP involve in the development of pathological conditions of oxidative stress and aging (Calabrese *et al.*, 2012).

The oxidative stress was also reduced in IPC-mediated cardio-protection in OCD challenged rat heart. The pretreatment of DDZ increases the effect of IPC on increases oxidative stress, but when given

quercetin with DDZ the cardio-protective effect reduced in OCD challenged rat heart. Also, in this investigation, the IPC-mediated cardio-protection by caveolin inhibitor in OCD rat heart was remarkably diminished by pretreatment of quercetin. So the phenomenon involve in reduction of IPC interceded myocardial protection in OCD rat may be reduced by expression of HSP-72, in response to stress. The results showed that HSP-72follows the pathway of caveolin and act a significant function in cardio-protection.

CONCLUSIONS

The result of this study revealed that caveolin inhibitor DDZ enhances the protection of myocardium through IPC in OCD rats and this result may be abolished by quercetin which lowers the synthesis of HSP-72 and acts on the pathway of caveolin to a major role in IPC-mediated cardio-protection in OCD challenged rats.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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