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Article

Role of ACE and ACE-2 in abrogated cardioprotective effect of ischemic preconditioning in ovariectomized rat heart

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Ischemic heart disease is the leading cause of death in postmenopausal women. The activity of heart ACE increases whereas the activity of ACE-2 decreases after menopause. The present study was designed to investigate the role of ACE and ACE-2 in the abrogated cardioprotective effect of IPC in OVX rat heart. The heart was isolated from OVX rat and mounted on Langendorff's apparatus for giving intermittent cycles of IPC. The infarct size was estimated using TTC stain, and coronary effluent was analyzed for LDH, CK-MB, and nitrite release. IPC induced cardioprotection was significantly attenuated in the ovariectomized rat heart as compared to the normal rat heart. However, this attenuated cardioprotection was significantly restored by perfusion of DIZE, an ACE-2 activator, and captopril, an ACE inhibitor, alone or in combination noted in terms of decrease in myocardial infarct size, the release of LDH and CK-MB, and also increase in the release of NO as compared to untreated OVX rat heart. Thus, it is suggested that DIZE and captopril, alone or in combination restore the attenuated cardioprotective effect of IPC in OVX rat heart which is due to an increase in ACE-2 activity and decrease in ACE activity after treatment.

Keywords: Ovariectomy. Captopril. Diminazene aceturate. Nitric oxide. Ischemic Preconditioning.

INTRODUCTION

Ischemic heart disease (IHD) has been identified as the world's leading cause of mortality and morbidity (Murray, Lopez, 1997). Reperfusion of the ischemic myocardium is mandatory for the restoration of the normal functioning of the myocardium (Topol, Califf, Vandormael, 1992). However, abrupt reperfusion of an ischemic heart produces further damage of the myocardium, described as ischemic/reperfusion (I/R) injury (Baxter, Ebrahim, 2002; Piper, Abdullah, Schafer, 2004). Ischemic preconditioning (IPC) is a powerful endogenous protective phenomenon that is used to protect the myocardium from ischemic insults, and it comprises short intermittent cycles of sublethal ischemia and reperfusion before the subsequently prolonged ischemic insult (Murray, Jenning, Reimer, 1986). IPC produces cardioprotection by various mechanisms (Stokoe *et al*., 1997; Ferdinandy, Schulz, Baxter, 2007; Prendes *et al*., 2007; Garg *et al*., 2010 Goyal *et al*., 2016; Charan *et al*., 2016). However, the cardioprotective effect of IPC gets attenuated in certain pathological conditions such as diabetes mellitus (Ajmani *et al*., 2011; Charan *et al*., 2016), hyperlipidaemia (Yadav, Singh, Sharma, 2010), hypertension (Snoeckx *et al*., 1986; Snoeckx *et al*., 1996), aging (Abete *et al*., 1996; Liu *et al*., 2004), hypertrophy (Singh *et al*., 2008), obesity (Sasaki *et al*., 2007), and estrogen deficiency (Goyal, Semwal, Yadav, 2016). Estrogen deficiency is one of the major risk factors of ischemic heart disease (Shinmura, Nagai, Tamaki, 2008). It is well documented that men are more susceptible to the risk of IHD than women (Barrett-Connor, 1997). But after menopause, the risk of IHD in women reaches the same level as in men of the same age (Barrett-Connor, 1997; Clarkson, Cline, Williams, 1997). Hence there is an urge to detect a possible mechanism involved in the abrogated cardioprotective effect of IPC in ovariectomized (OVX) rat heart.

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The heart renin-angiotensin system (HRAS) plays an important role in the homeostasis of the cardiovascular system (David, Kenneth, 1999). It has been demonstrated that an increase in activity of heart angiotensin-converting enzyme (ACE) stimulates cardiomyocytes hypertrophy and fibroblast proliferation that lead to left ventricular hypertrophy, arrhythmia, heart failure, increased myocardial infarct size, and ultimately cardiac death (Alderman, 2004). Further, it has been well suggested that angiotensin-converting enzyme-2 (ACE-2), a new component of RAS exhibit an opposing function to the ACE (Ferreira, Santos, Almeida, 2001). The equilibrium of both enzymes is necessary to maintain the homeostasis of the cardiovascular system. This contention is supported by the other laboratories that the administration of ACE inhibitors and ACE-2 activators decrease the myocardial infarct size as well as decrease the release of markers of myocardial injury (LDH and CK-MB) (Martinez, Molina, 2003; Katovich, Raizada, 2013; Fraga-Silva *et al*., 2015). However, it has also been documented that the pretreatment of ACE inhibitors and ACE-2 activators produce cardioprotection by facilitating the release of nitric oxide (NO) (Comini *et al*., 2007; Brancaleone, Bucci, 2008; Fraga-Silva *et al*., 2015). Further, it has been well known that NO produces IPC mediated cardioprotection, and their down-regulation abrogates the cardioprotective effect of IPC (Ajmani *et al*., 2011; Goyal, Semwal, Yadav, 2016).

The cardioprotective effect of IPC gets attenuated in estrogen-deficient or OVX rat hearts (Goyal, Semwal, Yadav, 2016). It has been documented that the level of ACE gets upregulated whereas the level of ACE-2 gets downregulated during estrogen deficiency which further decreases the release of NO which is known to produce IPC mediated cardioprotection (Lindsey *et al*., 2009; Goyal, Semwal, Yadav, 2016). Therefore, the present study has been designed to investigate the role of ACE and ACE-2 in the modulation of the cardioprotective effect of ischemic preconditioning in the ovariectomized rat heart.

MATERIAL AND METHODS

Female Wistar rats weighing about 180-250 g were kept in the animal house and provided 12h light, and the 12h dark cycle was employed in this study. They were fed on a standard chow diet (wheat flour 22.5%, roasted Bengal gram powder 60%, skimmed milk powder 5%, casein 4%, refined oil 4%, salt mixture with starch 4% and vitamin and choline mixture 0.5%) and provided water ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee (GLAIPR/CPCSEA/IAEC/2016/P.Col/R15) in accordance with the national guidelines on the use of laboratory animals.

Drugs and chemicals

Captopril (ACE inhibitor) (100µM/L) and Diminazene aceturate (DIZE) (ACE-2 activator) (26.35mg/L) (Sigma Aldrich [P] Ltd., Bangalore, India) were dissolved in Kreb's-Henseleit (K-H) buffer and then perfused to the isolated heart in four cycles of reperfusion. All other reagents used in this study were of analytical grade and always freshly prepared before use.

Induction of experimental ovariectomy

Total seven groups have been used in the present study; each group consists of six female Wistar rats. Female rats were anesthetized with pentobarbitone (45 mg/Kg i.p.). Ovariectomy was performed by making a peritoneal incision of 0.4–0.6 cm on the middle part of the abdomen slightly towards the right. Ovary and associated fat were easily located and exteriorized by gentle retraction. Ovaries along with the uterus were pulled out, and the suture was applied at the end of the uterus and beginning of the ovary. Ovaries were removed, the uterus was pushed back, and incisions were sutured in layers. Neomycin antibiotic powder was applied twice daily on wounds for one week, and animals were allowed to recover for four weeks (Goyal, Semwal, Yadav, 2016).

Isolated rat heart preparation

Rats were administered heparin (500 IU/L, i.p.) (Gland Pharma Ltd., Hyderabad, India) 20 min prior to sacrifice by cervical dislocation, and the Heart was rapidly excised and was immediately mounted on Langendorff's apparatus (Langendorff, 1895). The

heart was enclosed in a double-walled jacket, and the temperature was maintained at 37 ºC by circulating warm water. The isolated heart was retrogradely perfused at a constant pressure of 80 mmHg and coronary flow rate of 7-9 mL/min with Kreb's Henseleit (K-H) buffer (NaCl 118 mM; KCl 4.7 mM; $CaCl_2$ 2.5 mM; $MgSO_4$.7H 1.2 mM; KH_2PO_4 1.2 mM; $C_6H_{12}O_6$ 11 mM), pH 7.4, maintained at 37 ºC bubbled with 95% O_2 and 5% CO_2. Global ischemia was produced for 30 min by blocking the inflow of Kreb's Henseleit solution, which was 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), creatine kinase (CK-MB), and nitrite release (Skrzypiec-Spring, Grotthus, Szela, 2007; Ajmani *et al*., 2011).

Assessment of myocardial injury

The myocardial injury was assessed by the estimation of lactate dehydrogenase (LDH) and creatinine kinase-MB (CK-MB) in the coronary effluent (Skrzypiec-Spring, Grotthus, Szela, 2007; Yadav, Singh, Sharma, 2010) by using a commercially available kit (Coral clinical system, Goa, India). Values are expressed in international unit IU per liter (IU/L).

Myocardial infarct size measurement

The heart was removed from Langendorff's apparatus. Both the auricles and root of the aorta were excised, and ventricles were kept overnight at -4 ºC temperature. Frozen ventricles were sliced into uniform sections of about 2-3 mm thickness and incubated at 37 ºC for 30 min in 1% w/v triphenyltetrazolium chloride stain (TTC stain) in 0.2 M Tris-chloride buffer, pH 7.4 (Fishbein, Meerbaum, Rit, 1981). The viable cells were stained brick red due to the conversion of TTC to red formazone pigment by NADH and dehydrogenase enzyme (Nachlas, Schnitka, 1963). While the infarcted cells have lost the enzyme and co-factor and thus remained dull yellow or unstained. Infarct size was measured macroscopically and expressed as a percentage of average infracted ventricular volume (Klein, Pushman, Schaper, 1981; Chopra, Singh, Kaul, 1992).

Nitrite Estimation

Unlike NO, nitrite can be measured easily, and nitrite concentrations can be used to infer levels of NO production (Marletta, Yoon, Iyenger, 1988). Nitrite release in coronary effluent was measured (Szabo, Thiemermann, Vane, 1993; Szabo, Wu, Mitchell, 1993). 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 a spectrophotometer. Nitrite concentration was calculated by comparison with spectrophotometer readings of the standard solution of sodium nitrite prepared in K-H buffer (Ajmani *et al*., 2011). Results were expressed as micromoles per litre (μM/L).

Experimental Protocol

The present study was conducted on seven groups, and each group comprised of six rats. The diagrammatic representation of the experimental protocol is shown in Figure 1.

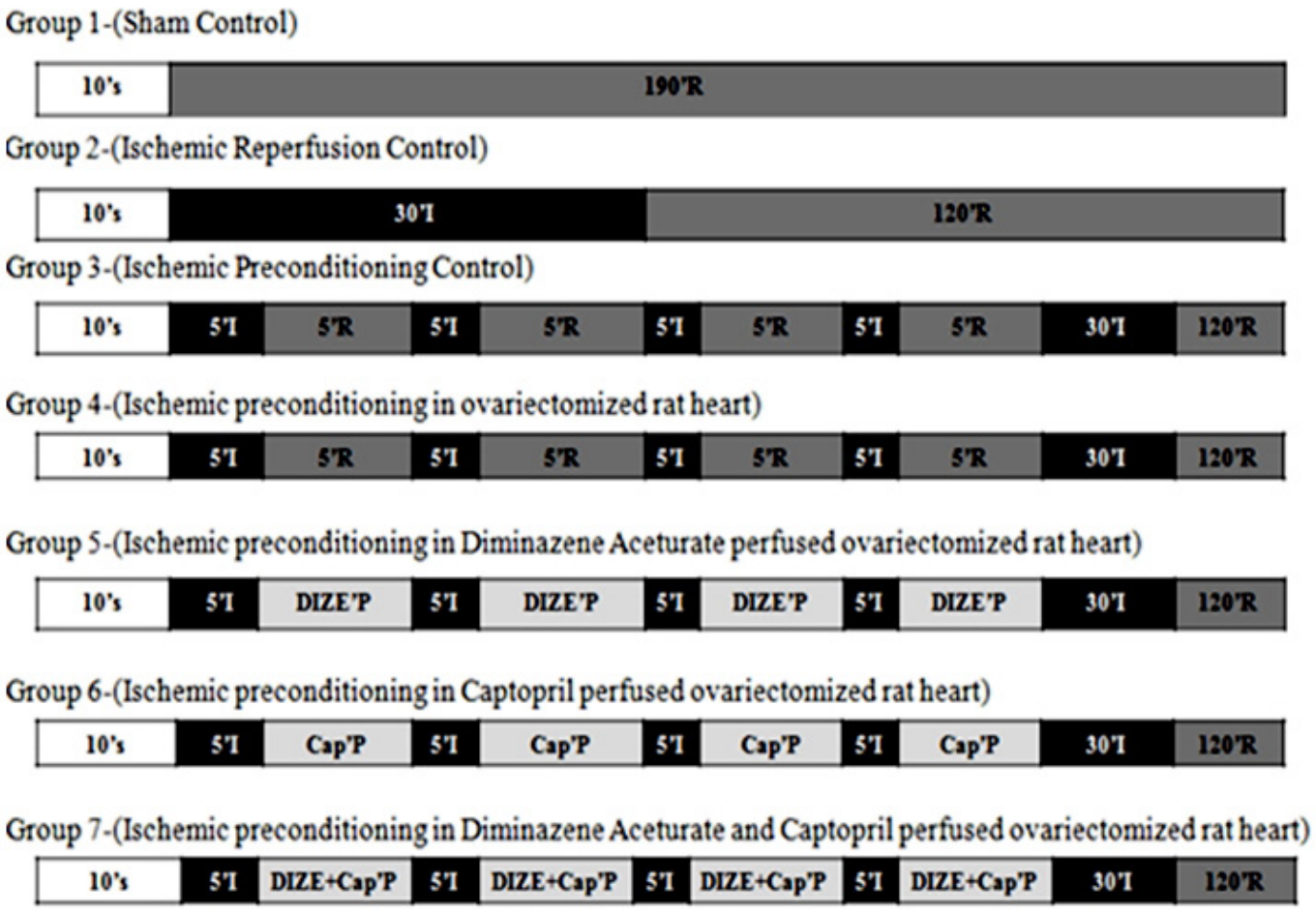

FIGURE 1 - Diagrammatic representation of experimental protocol. S, P, I, R, DIZE, Cap denote stabilization, perfusion, ischemia, reperfusion, diminazene aceturate, captopril.

Group I - (Sham control; n=6): Isolated rat heart preparation was stabilized for 10 min and then perfused continuously with K-H buffer solution for 190 min without subjecting them to global ischemia.

Group II - (Ischemia/reperfusion Control; n=6): Isolated rat heart preparation was allowed to stabilize for 10 min then it was subjected to 30 min global ischemia followed by 120 min of reperfusion.

Group III - (Ischemic preconditioning control; n=6): Isolated rat heart preparation was allowed to stabilize for 10 min and subjected to four cycles of ischemic preconditioning, each cycle comprised of 5 min global ischemia followed by 5 min reperfusion with K-H buffer solution. Then the preparation was subjected to 30 min global ischemia followed by 120 min of reperfusion.

Group IV - (Ischemic preconditioning in ovariectomized rat heart; n=6): Isolated heart preparation from ovariectomized rat was allowed to stabilize for 10 min and subjected to four cycles of ischemic preconditioning as described earlier in group III.

Group V - (Ischemic preconditioning in DIZE (26.35 mg/L) perfused ovariectomized rat heart; n=6): Isolated rat heart preparation was allowed to stabilize for 10 min and perfused with DIZE (26.35 mg/L) in each cycle of 5 min reperfusion. The rest of the protocol was the same as described in group III.

Group VI - (Ischemic preconditioning in Captopril (100µM/L) perfused ovariectomized rat heart; n=6): Isolated rat heart preparation was allowed to stabilize for 10 min and perfused with captopril (100µM/L) in each cycle of 5 min reperfusion. The rest of the protocol was the same as described in group III.

Group VII - (Ischemic preconditioning in DIZE (26.35 mg/L) and Captopril (100µM/L) perfused ovariectomized rat heart; n=6): Isolated rat heart preparation was allowed to stabilize for 10 min and perfused with DIZE (26.35 mg/L) and captopril (100µM/L) in each cycle of 5 min reperfusion. The rest of the protocol was the same as described in group III.

Statistical Analysis

All values were expressed as mean ± S.D (standard deviation). Statistical analysis was performed using Sigmastat Software. The data obtained from the various groups were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *p*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Effect of IPC and pharmacological intervention on myocardial infarct size

Global ischemia for 30 min, followed by reperfusion of 120 min significantly increased the myocardial infarct size as compared to sham control. Four cycles of 5 min ischemia and 5 min reperfusion (IPC) were sufficient to markedly prevent I/R induced increase in myocardial infarct size in normal rat heart but not in ovariectomized rat heart. However, perfusion of DIZE (ACE-2 Activator) (26.35mg/L) and Captopril (ACE Inhibitor) (100µM/L) alone or in combination in each cycle of 5 min of reperfusion significantly restored the IPC induced decrease in myocardial infarct size in ovariectomized rat heart (Figure 2).

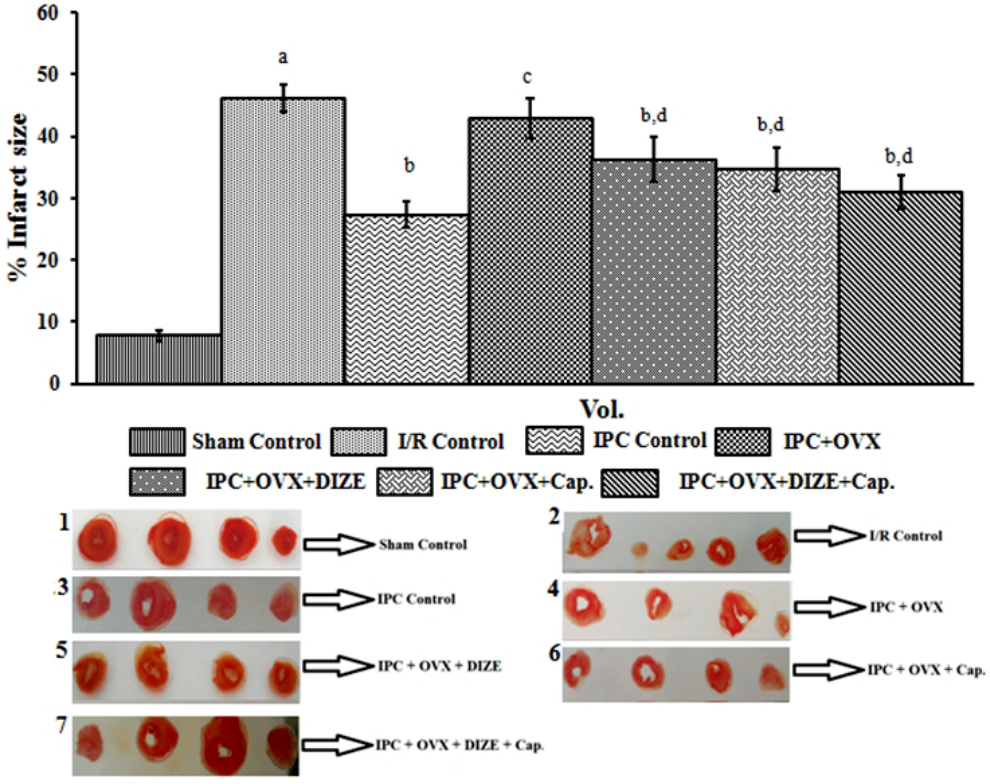

FIGURE 2 - Myocardial infarct size with Images of the TTC-stained sections of the heart (1-7); Effect of I/R on myocardial infarct size, the effect of ischemic preconditioning (IPC) on myocardial infarct size in normal and OVX rat heart, the effect of DIZE perfusion on myocardial infarct size in ovariectomized rat heart and effect of Cap. alone or in combination with DIZE on myocardial infarct size in OVX rat heart. I/R denotes ischemia-reperfusion; IPC denotes ischemic preconditioning; OVX denotes ovariectomy; DIZE denotes diminazene aceturate; Cap. denotes captopril. 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 normal rat heart; d = p< 0.05 vs. IPC in OVX rat heart.

Effect of IPC and pharmacological intervention on the release of LDH and CK-MB

Global ischemia for 30 min, followed by reperfusion of 120 min significantly increased the release of LDH and CK-MB as compared to sham control. Four cycles of 5 min ischemia and 5 min reperfusion were sufficient to markedly prevent I/R induced increase in the release of LDH and CK-MB in normal rat heart but not in ovariectomized rat heart. However, perfusion of DIZE (ACE-2 Activator) (26.35mg/L) and Captopril (ACE Inhibitor) (100µM/L) alone or in combination in each cycle of 5 min of reperfusion significantly restored the IPC induced decrease in the release of LDH and CK-MB in ovariectomized rat heart (Figure 3, 4).

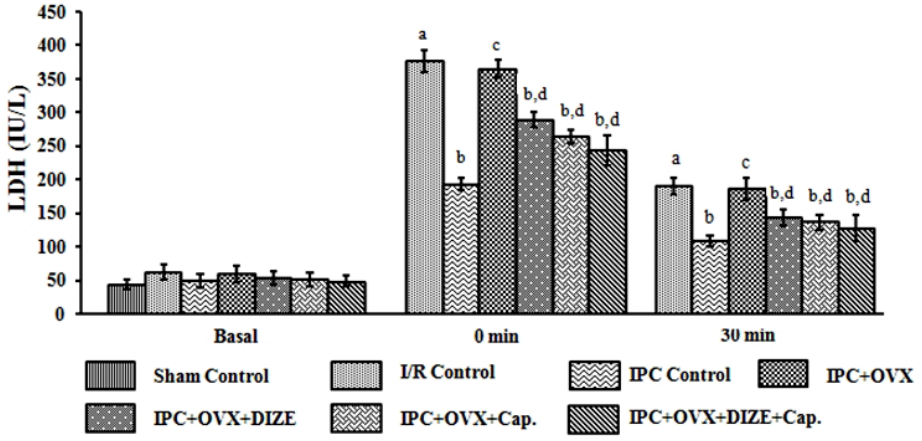

FIGURE 3 - Effect of I/R on the release of LDH, effect of ischemic preconditioning (IPC) on the release of LDH in normal and OVX rat heart, effect of DIZE perfusion on the release of LDH in ovariectomized rat heart and effect of Cap. alone or in combination with DIZE on the release of LDH in OVX rat heart. I/R denotes ischemia reperfusion; IPC denotes ischemic preconditioning; OVX denotes ovariectomy; DIZE denotes diminazene aceturate; Cap. denotes captopril. 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 normal rat heart; d = p< 0.05 vs. IPC in OVX rat heart.

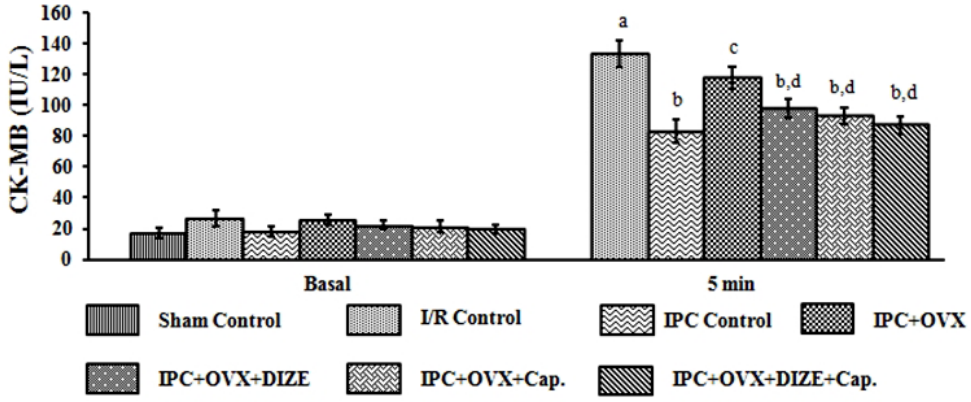

FIGURE 4 - Effect of I/R on the release of CK-MB, effect of ischemic preconditioning (IPC) on the release of CK-MB in normal and OVX rat heart, effect of DIZE perfusion on the release of CK-MB in ovariectomized rat heart and effect of Cap. alone or in combination with DIZE on the release of CK-MB in OVX rat heart. I/R denotes ischemia reperfusion; IPC denotes ischemic preconditioning; OVX denotes ovariectomy; DIZE denotes diminazene aceturate; Cap. denotes captopril. 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 normal rat heart; d = p< 0.05 vs. IPC in OVX rat heart.

Effect of IPC and pharmacological intervention on the release of nitrite

The release of nitrite in coronary effluent was noted to be significantly reduced in ovariectomized rat heart when compared to normal rat heart. Perfusion of DIZE (ACE-2 Activator) (26.35mg/L) and Captopril (ACE Inhibitor) (100µM/L) alone or in combination in each cycle of 5 min of reperfusion significantly increased the release of nitrite in ovariectomized rat heart when compared to untreated ovariectomized rat heart (Figure 5).

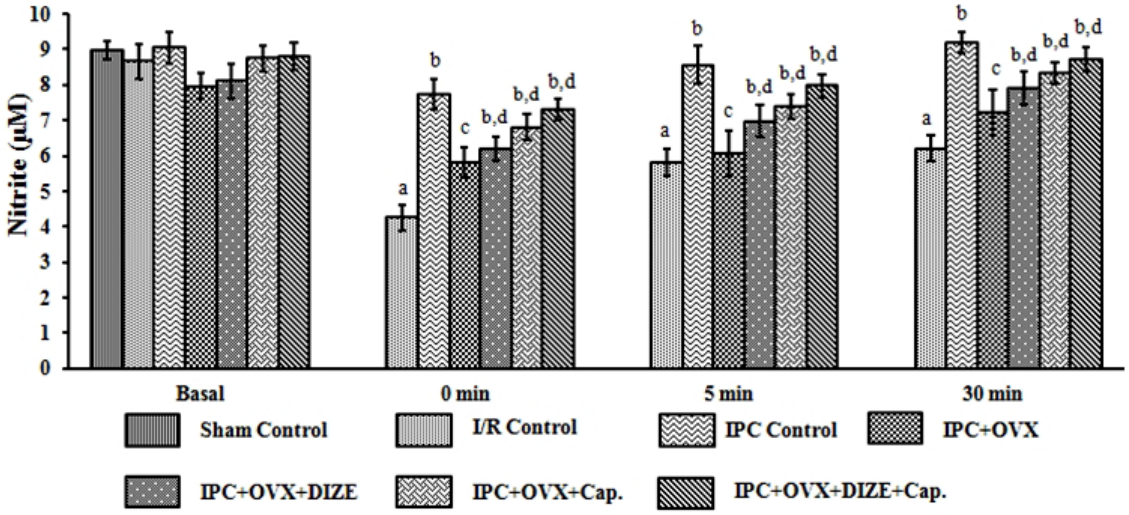

FIGURE 5 - Effect of I/R on the release of nitrite, effect of ischemic preconditioning (IPC) on the release of nitrite in normal and OVX rat heart, effect of DIZE perfusion on the release of nitrite in ovariectomized rat heart and effect of Cap. alone or in combination with DIZE on the release of nitrite in OVX rat heart. I/R denotes ischemia reperfusion; IPC denotes ischemic preconditioning; OVX denotes ovariectomy; DIZE denotes diminazene aceturate; Cap. denotes captopril. 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 normal rat heart; d = p< 0.05 vs. IPC in OVX rat heart.

DISCUSSION

The extent of release of LDH and CK-MB is directly correlated with the degree of damage of myocardium during ischemia and reperfusion (I/R) (Wang, Cherednichenko, Hernandez, 2001). An increase in myocardial infarct size elevated levels of LDH and CK-MB and decreased level of NO in coronary effluent are the indicators of ischemia/reperfusion-induced myocardial injury (Yadav, Singh, Sharma, 2010; Kaur, Parikh, Sharma, 1997). In the present study, 30 min of global ischemia and 120 min of reperfusion increased the myocardial infarct size, release of LDH and CK-MB, and decreased the release of nitrite in coronary effluent of normal rat heart which is consistent with our earlier finding (Goyal, Semwal, Yadav, 2016; Charan *et al*., 2016). However, four episodes of 5 min of ischemia and 5 min of reperfusion were sufficient to significantly attenuate the I/R induced increased myocardial infarct size, the release of LDH and CK-MB and decreased the level of NO in coronary effluent of normal rat heart. This observation is consistent with our previous reports (Goyal, Semwal, Yadav, 2016).

The HRAS is an important system for cardiac functions, and it exerts many actions through its components to regulate cardiac physiology (David, Kenneth, 1999). It has been documented that ACE and ACE-2 both enzymes maintain the homeostasis in the cardiovascular system and further it has been well suggested that an imbalance between ACE and ACE-

2 for a longer duration cause several cardiovascular complications (Ferreira, Santos, Almeida, 2001; Sakima, Averill, Gallagher, 2005; Diz, Garcia-Espinosa, Gallagher, 2008). An increase in ACE activity and decrease in ACE-2 activity exert deleterious effects on cardiovascular function in ischemia/reperfusion challenged heart (Degraeff *et al*., 1988; Qi *et al*., 2013). A galaxy of experimental studies reported the cardioprotective activity of ACE inhibitors and ACE-2 activators against I/R injury (Degraeff *et al*., 1988; Pfeffer, Braunwald, Moy, 1992; Qi *et al*., 2013). This contention is supported by the fact that I/R injury after regional or global ischemia involves damage to the cardiomyocytes, vascular smooth muscles, and endothelial cells and the administration of ACE inhibitors and ACE-2 activators protect the myocardium from I/R injury and limit the infarct size of the myocardium (Pfeffer, Braunwald, Moy, 1992; Martinez, Molina, 2003; Qi *et al*., 2013). Further, it has been reported that ACE inhibitors and ACE-2 activators increase the release of NO and limit the infarct size (Zhang *et al*., 1997; Fraga-Silva *et al*., 2015). The activity of ACE is upregulated while the activity of ACE-2 is downregulated in estrogen deficiency which further decreases the level of nitric oxide (Lindsey *et al*., 2009; Pereira, Bertolami, Faludi, 2013).

In our study, IPC induced cardioprotection and the release of nitrite in OVX rats was significantly reduced as compared to normal rat heart, which is supported by the finding of our laboratory (Goyal, Semwal, Yadav, 2016). This may be due to increased activity of ACE and decreased activity of ACE-2.

Captopril, an ACE inhibitor has been noted to inhibit the ACE activity (Brown, aughan, 1998) and facilitates the IPC induced release of NO (Zhang *et al*., 1997; Tian *et al*., 2015). In the present study, perfusion of captopril restored the cardioprotective effect of IPC and increased the release of NO in the OVX rat heart. Further, the perfusion of diminazene aceturate (DIZE), an ACE-2 activator also decreased the myocardial infarct size, release of LDH and CK-MB in the coronary effluent of OVX rat heart as compared to untreated OVX rat heart. These findings also support that the infarct size-limiting effect of captopril and DIZE is mediated through NO. Furthermore, co-perfusion of captopril and DIZE was unable to produce any significant cardioprotective effect of IPC as compared to individual perfusion of both the drugs in OVX rat heart noted in terms of infarct size, the release of LDH, CK-MB, and nitrite in coronary effluent.

Our data indicate that individual treatment of captopril and DIZE with IPC protect the myocardium from estrogen deficiency-induced injury. So, the treatment of hypoestrogenism related patients undergoing cardiopulmonary bypass with captopril or DIZE could be a beneficial adjunctive for myocardial protection during open heart surgery.

CONCLUSION

On the basis of the above discussion, it is concluded that the perfusion of ACE inhibitor and ACE-2 activator restore the attenuated cardioprotective effect of ischemic preconditioning in ovariectomized rat heart. This observed cardioprotective effect is due to the decreased ACE activity or increased ACE-2 activity.

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CONTRIBUTION OF AUTHORS

All authors contributed equally to this work. *J.K.G.*(Supervisor) and *A.G.* (Internal supportive member of the study) developed the concept and designed experiment. *V.K.* experimented on Langendorff's apparatus. *A.G.* and *J.K.G.* carried out the analysis of infarct size and data of other parameters (LDH, CK-MB, and Nitrite). *V.K.* prepared the manuscript.

FINANCIAL/SOURCE OF FUNDS STATEMENT

There are no funding sources.

CONFLICT OF INTEREST

All authors have no conflict of interest to declare.

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