Article

Ceiling effect of Postconditioning and Atrial Natriuretic Peptide in Cardioprotection against Ischemia Reperfusion Injury in Ovariectomized rat hearts

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Ischemic postconditioning (IPTC) brings cardioprotection endogenously, Atrial natriuretic peptide (ANP) produces the same effect. It happens due to down expression of endothelial nitric oxide synthase (eNOS). Thus, experimental protocol associating IPTC has been formulated to find the role of ANP in the cardioprotection of heart in OVX rats. For this experiment, heart was isolated from OVX rat and held tightly on Langendorff's apparatus in a manner that ischemia of 30 min and reperfusion of 120 min were also given. Simultaneously, IPTC with four cycles of 5 min ischemia and 5 min reperfusion of each was applied. Parameters like size of myocardial infarct, levels of lactate dehydrogenase (LDH) and release of creatine kinase-MB (CK-MB) in coronary effluent were noted after each stage of experiment for ensuring the extent of myocardial injury. Some significant changes were also seen in the histopathology of cardiovascular tissues. The cardio-protection has been made by four cycles of IPTC. It was confirmed by decline in the size of myocardial infarct. It diminishes the release of LDH and CK-MB in heart of OVX rat. Thus, IPTC induces cardio-protection in the isolated heart from OVX rat. Perfusion of ANP associating with IPTC favors the cardioprotection which is further confirmed by rise in the NO release and heart rate. The level of myocardial damage changes using IPTC, IPTC+OVX, IPTC+OVX+ANP, IPTC+ OVX+ANP+L-NAME and other groups were observed significantly and were found to be less than those in I/R control group. Thus, it is recommended that ANP involving IPTC restores attenuated cardio-protection in OVX rat heart. Therefore, Post-conditioning is useful in various clinical aspects.

Keywords: Ovariectomy. Atrial natriuretic peptide. Nitric oxide. Ischemic postconditioning. Glibenclamide.

INTRODUCTION

In many cases, the cause of morbidity is worldwide due to occurrence of ischemic heart disease (Murray *et al.*, 1997). The normal performance of heart is returned necessarily by reperfusion of an ischemic heart (Topol *et al.*, 1992). The rapid reperfusion of a heart in ischemic conditions recovers a progression of myocardium damage caused by ischemia reperfusion injury (I/R injury) (Popma *et al.*, 1994; Kloner, 1993). A phenomenon named ischemic postconditioning (IPTC) which includes some short-lived cycles of ischemia-reperfusion changes ischemic episode of reperfusion stage. Ultimately, it causes a decline in myocardial injury (Vinten-Johansen et al., 2005; Zhao *et al.*, 2003).

Estrogens are secreted mainly from the ovaries in significant quantities, whereas adrenal cortices secrete in minor quantities. β-estradiol is considered to be the chief estrogen, although estrogenic effects of the oestrone are more significant (Mittal *et al.*, 2009). These estrogens

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are responsible for secondary sexual characteristics of female such as breast development, regulate the menstrual cycle and recover the uterus rupturing during various changes in the endometrial wall in pregnancy (Ferenczy*et al.*, 1979). Women are less susceptible to *ischemic heart disease* (IHD) than men. The risk of IHD in women came to the same level from men of same age. This disease is a greater risk factor among women after menopause (Barrett-Connor, 1997; Clarkson *et al.*, 1997).

The IPTC provokes cardio-protection in various ways which include activation of PI-3K-Akt pathway, adenosine activation (Philipp *et al.*, 2006); bradykinin function (Penna *etal.*, 2007) and contributory role of erythropoietin (Hausenloy *et al.*, 2005).

Another routes of imparting function revealed that generation of nitric oxide (NO) and opening of mitochondrial ATP-sensitive potassium channel (mito-K_{ATP}) mediate cardioprotection (Selzner *et al.*, 2012). The cardio-protective effect of IPTC is weakened in different physiological and disease conditions like hyper-homocysteinia (Rohilla *et al.*, 2009), hyperlipidemia (Zhao *et al.*, 2009), estrogen inadequacy (Couvreur *et al.*, 2009) and diabetes mellitus (Yin *et al.*, 2012).

Atrial natriuretic peptide (ANP) is a polypeptide hormone of heart which has prominent dilatory action on vessels (Dietz, 2005; Ichiki *et al.*, 2017). ANP receptors (ANPR) have three categories like natriuretic peptide receptor A (NPR-A), natriuretic peptide receptor B (NPR-B) and natriuretic peptide receptor C (NPR-C). These receptors work by means of initiation of guanylate cyclase that is found to make safe by augmenting cGMP synthesis against reoxygenated-prompted hypercontracture in detached cardiomyocytes (Nishikimi *et al.*, 2006; Sangwa *et al.*, 2004).

The activation of endothelial nitric oxide synthase (eNOS) is triggered by the action of ANP which results to elevate the production of NO (Fujii *et al.*, 2012). The injuries of vascular smooth muscle, cardiomyocytes and endothelial cells are associated with I/R injury caused by global or regional ischemia. The myocardium is protected using perfusion of ANP from the I/R injury at the time of reperfusion (Sangwa *et al.*, 2004; Fujii *et al.*, 2012). Estrogen has crucial role in the accumulation of adipose tissue during later half of

female's life cycle (Mattsson *et al.*, 2007). In every postmenopausal woman, the fat mass rises drastically which results to the occurrence of chronic diseases like hyperlipidaemia and hypertension (Munoz *et al.*, 2002). Estrogen deficiency is the main risk factor in ischemic heart disease because cardio-protection is down regulated in estrogen deficiency, thus weakens the cardio-protection (Mc Neill *et al.*, 2002).

In case of estrogen inadequacy, ANP cause a decrease in generation of NO due to deactivation of eNOS. Since, NO production encourages IPTC mediated cardioprotection (Zaman *et al.*, 2014; Jeddi *et al.*, 2016). Thus, this research work has been proposed to discover ANP effects in the modulation of cardio-protection mediating IPTC in normal as well as OVX rats.

MATERIAL AND METHODS

Drugs and chemicals

ANP (Atrial natriuretic peptide) and L-NAME (N-nitro L-arginine methyl ester) was procured from Sigma-Aldrich [P] Ltd. Delhi, India. While, Glibenclamide was obtained from Medirose Drug and Pharmaceutical Pvt. Ltd, Kangra, Himachal Pradesh, India. A solution of ANP (0.1 µM/L) was transferred to Kreb's Henseleit (K-H) buffer. The isolated female rat heart is then perfused with the prepared solution in four cycles of reperfusion, 30 days after the ovariectomy. L-NAME (100 µM/L) and Glibenclamide (10 µM/L) were prepared in distilled water and K-H buffer added to it. High purity chemicals and freshly prepared reagents were used in the experiment.

Animals

Each animal weighed about 180-250 g grouped from female Wister rats. Each group contained six animals. They were kept in the animal house giving an exposure of 12 h light and 12 h dark before beginning of experiment. A standard chow diet contains wheat flour 22.5%, skimmed drain powder 5%, casein 4%, salt blend with 4% starch, simmered Bengal gram powder 60%, refined oil 4%, vitamins and choline blend 0.5%.

This diet was supplied to all animals and provided water *ad libitum*.

Institutional Animal Ethical Committee has approved this experimental protocol according to national guidelines of laboratory animals.

Surgical Preparation

Induction of experimental ovariectomy

An anesthetic like Pentobarbitone (45 mg/kg, i.p.) was given to female rats. The anesthetized rat was kept ready for surgical operation in way that dorsal surface lies in lower side. The zone was indicated for surgical operation then it was shaved for removing hairs. The ethanol was used for cleaning of surgical area. An incision was made around 0.4-0.6 cm on transverse peritoneal region with surgical scalpel blade on marginally towards right of mid stomach area. Then, a peritoneal cavity was identified, fallopian tube and ovary surrounded by fat were recognized and fat tissue pulled away. Further, fat and ovary were effectively exteriorized by delicate withdrawal. The same surgical method was also repeated for the left ovary.

After identification of ovary in the abdominal cavity, the peri-ovarian fat was withdrawn safely, sidewise from the incision site of ovary. It may re-implant to carry on its typical function. Now, sutures were connected between end of the uterus and start of ovary. The ovaries were withdrawn from peritoneal cavity and uterine curvature also came back to the cavity. For this, two layers (muscle and skin) were formed from the cut which was bound closely using absorbable sutures.

After suturing, Povidone-iodine was spread over skin area to prevent the probable skin infections. More specifically, the aseptic conditions were maintained through various strategies to defend animals after surgery. Thus, the animals were kept for recovery in a month (Khajuria *et al.*, 2012).The rats were housed entirely in the cages which should be clean, dry and with sterilized cotton fabric bedding to provide extra comfort. A warmth conditions were maintained for some weeks and animal area was be free from contamination (Figure 1).
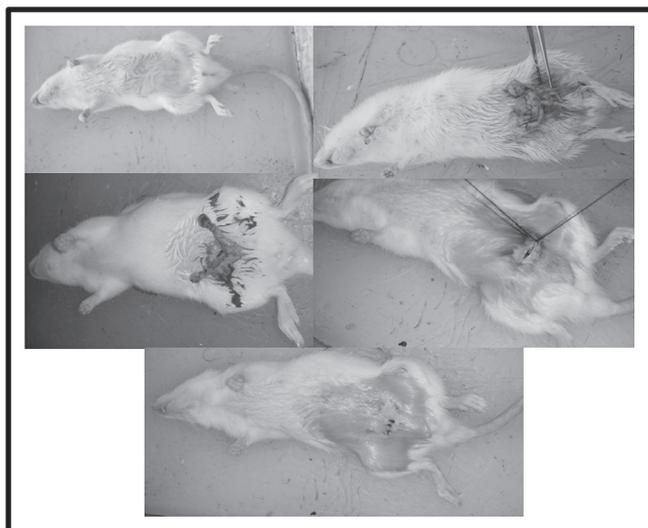

FIGURE 1 - Surgical procedure of ovariectomy**.**

Isolated rat heart preparation

The female Wister rats were sacrificed after 20 min of heparin (500 IU/L, i.p.) administration which was purchased from Gland Pharma Ltd., Hyderabad, India. The sacrifice of animal was done by cervical dislocation method. After surgery of animal, the isolated heart was promptly held on Langendorff's apparatus (Langendorff *et al.*, 1895). A constant pressure was managed to 80 mmHg when isolated heart was conservatively perfused. The Krebs-Henseleit (K-H) buffer (CaCl$_2$ 2.5 mM; KCl 4.7 mM; NaCl 118 mM; C$_6$H$_{12}$O$_6$ 11 mM; MgSO$_4$ 7H$_2$O 1.2 mM; KH$_2$PO$_4$1.2 mM) was prepared at 37.8 °C by maintaining pH 7.4. The conditions like 95% O$_2$ and 5% CO$_2$were managed to produce similar environment to living tissues. Hoffman's screw at temperature about 37.8°C was used to maintain 7–9 ml/min flow rate through hot water circulation as the heart encased in a two fold jacket. Global ischemia was produced for 30 min duration by stopping inflow of K-H buffer followed by 160 min reperfusion.

The ischemia of 5 min was produced during the cycle. The reperfusion of 5 min following IPTC action was given which was supported by inward flow of K-H buffer. Such cycles were repeated for four times. A coronary effluent was collected before producing 30 min of global ischemia and furthermore, 5 min ischemia was given carefully after fulfillment of four cycles of IPTC (Rohilla *et al.*, 2009). The estrogen effects were observed after for 30 of after surgical removal of ovary from the female rat

and compared to the sham controls. The removal of ovary was further confirmed by determining plasma estrogen levels both in normal rats and OVX rats. A plasma level of β-estradiol (6.8 pg/ml)is maintained in the bilateral ovariectomy condition.

Assessment of myocardial injury

The commercially available kits (Span Diagnostics Ltd., Surat, India) was used to estimate CK-MB and LDH in the coronary effluents which was estimated for assessing myocardial damage (Sharma *et al.*, 2000; Paulis *et al.*, 2013). The values of their estimations were expressed in terms of international units per litre (IU/L).

Evaluation of myocardial infarct size

The two auricles and base of aorta were detached from the heart. The ventricles were preserved at 4°C temperature for 12 hours. Uniform sized slices of 1-2 mm thickness were prepared from frozen ventricles and kept at 37°C for 30 min in 1% (w/v) triphenyl tetrazolium chloride (TTC) dye with every 0.2M Tris buffer of pH 7.4. Infarct bundle does not stain while ordinary myocardium stains to brick red color. This has been explained by TTC dye stain changed to red formazone by the action of both NADH and dehydrogenase enzyme (Figure 2). The volume method was mostly used to measure the infarct size (Yadav *et al.*, 2010; Fishbein *et al.*, 1981; Chopra *et al.*, 1992).
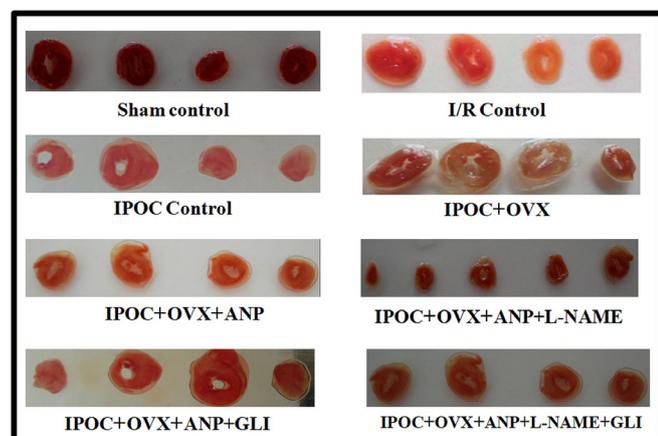

FIGURE 2 - Images of the TTC-stained transverse sections of heart**.**

Histopathological Examination

The heart was isolated and washed with ice cold saline at end of the experiment. The 10% buffered neutral formalin solution is used for fixing the tissue. After fixation, tissues were dipped in paraffin-wax. Five micrometer thick sections were cut. These were stained by haematoxylin stain following the use of eosin dye. The photomicrographs of histological slides were taken.

Estimation of nitric oxide/nirite level

NO generated stable nitrogen intermediate called nitrites were spontaneously deprivated. The level of NO generation can be determined by recognizing the nitrite concentration (Marletta *et al.*, 1988). So that, NO has been produced through increase of nitrite discharge in coronary effluent which was estimated (Szabo *et al.*, 1993a; 1993b) using Greiss reagent (equal concentration of 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride solution in water and 1% sulphanilamide in 5% phosphoric acid). A 0.5 ml of this reagent was added to 0.5 ml of coronary effluent and absorbance was noted at 550 nm using UV-visible spectrophotometer. Now, the concentration of nitrite was determined using sodium nitrite as standard which was prepared in K-H buffer solution (Sharma et al., 2000). Results were showed in terms of micromoles per liter (μM/L).

Assessment of Heart rate

The heart rate was measured by electrocardiogram during the study at basal, 0, 30, and 120 min (Pachauri *et al.*, 2017).

Experimental Protocol

This experimental protocol contained one arrangement of individual experiment (Figure 3). Animals were divided into eight group of six female rats (n=6). In every arrangement of experiment and designated as Sham Control, Ischemic reperfusion (I/R) Control, (IPTC) Control, IPTC in OVX rat heart, IPTC in the OVX rat heart by perfusion with ANP, IPTC in L-NAME and

ANP perfused OVX rat heart, IPTC in Glibenclamide
and ANP perfused OVX rat heart, IPTC in L-NAME,
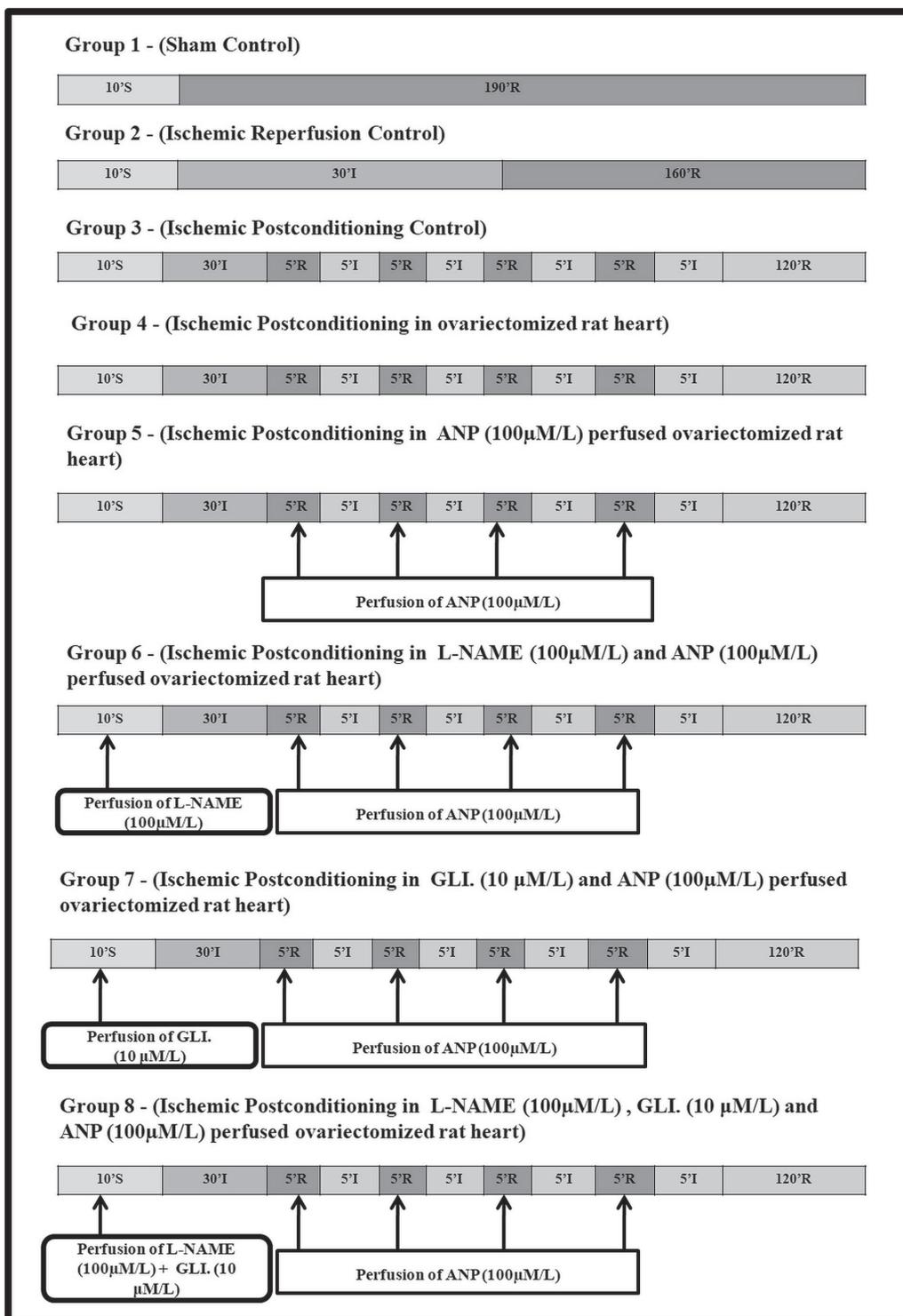Glibenclamide and ANP perfused OVX rat heart.

FIGURE 3 - Diagrammatic representation of experimental protocol. S, P, I, R, ANP, L-NAME and GLI denotes stabilization, perfusion, ischemia, reperfusion, atrial natriuretic peptide, N- nitro L-arginine methyl ester and Glibenclamide respectively.

Group 1: (Sham Control)

The stabilization of isolated female rat heart was exposed for about 10 min then perfused at this stage with K-H buffer for 190 min duration without giving any global ischemia and reperfusion.

Group 2: (Ischemic Reperfusion)

Similar way of stabilization was occurred in isolated female rat heart for 10 min. At that point heart was exposed to 30 min duration of global ischemia following 160 min duration of reperfusion.

Group 3: (Ischemic Postconditioning)

Isolated heart preparation of female rat was allowed to stabilize for a period of 10 min and exposed to 30 min duration of global ischemia on Langendroff's device.

This arrangement of preparation was permitted to come across four cycles of ischemia post conditioning. Each cycle comprised of 5 min ischemia and 5 min reperfusion followed by K-H buffer reperfusion of 120 min.

Group 4: (Ischemic Postconditioning in heart of ovariectomized rat)

Isolated heart preparation of OVX rat was allowed to stabilize for a period of 10 min and exposed to 30 min duration of global ischemia subsequently followed by four cycles of ischemic post-conditioning as depicted in group-3.

Group 5: (Ischemic Postconditioning in heart of ovariectomized rat perfused with ANP)

Isolated heart preparation of OVX rat was allowed to stabilize for a duration of 10 min and exposed to 30 min period of global ischemia at that point, preparation perfused with ANP (0.1 μM/L) in 5 min of each cycle of reperfusion of post conditioning taking after 120 min reperfusion with K-H buffer.

Group 6: (Ischemic Postconditioning in heart of ovariectomized rat perfused with L-NAME (100 μM/L) and ANP (0.1 μM/L)

The isolated heart was perfused with L-NAME (dissolved in 100 μM/L of K-H buffer) and then stabilized throughout

10 min duration. Rest of protocol was followed as same as depicted in group 5.

Group 7: (Ischemic Postconditioning in heart of ovariectomized rat perfused with Glibenclamide (10 μM/L) and ANP (0.1 μM/L)

The isolated heart was perfused with Glibenclamide (dissolved in 10μM/L K-H buffer) and then stabilized throughout 10 min duration. Rest of protocol was followed as same as depicted in group 5.

Group 8: (Ischemic Postconditioning in heart of ovariectomized rat perfused with L-NAME (100 μM/L), Glibenclamide (10 μM/L) and ANP (0.1 μM/L)

The heart preparation isolated from such female rat was perfused with Glibenclamide (10 μM/L K-H buffer) following L-NAME (100 μM/L K-H buffer) then being stabilized throughout 10 min duration. Rest of protocol was followed as same as depicted in group 5.

Statistical analysis

All biological data were interpreted in terms of Mean±SD which show significant outcomes. The various animal groups producing such data were statistically distinct using one-way analysis of variance (ANOVA) and two-way ANOVA followed by the Tukey's multiple comparison tests. P values<0.05 were considered to be statistically significant.

RESULTS

Effect of Ovariectomy on serum β-estradiol levels

In the OVX group, serum β-estradiol levels were significantly decreased. Since, adrenal glands and testes also produce small amount of female sex hormones, so that β-estradiol level did not reach to zero (Figure 4).
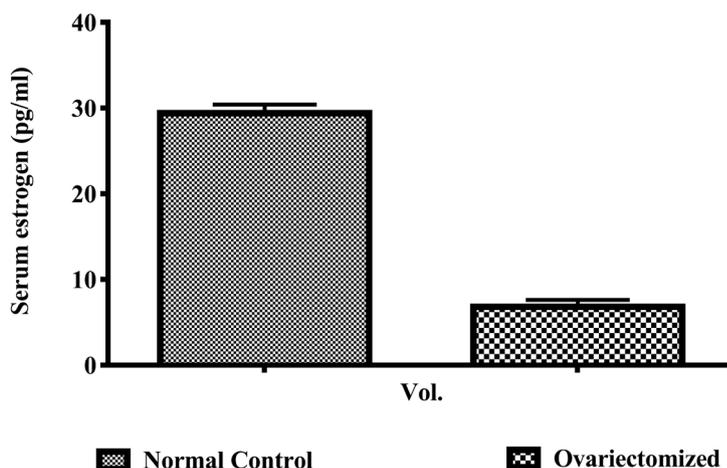
FIGURE 4 - Effect of ovariectomy on estrogen level.

Values are expressed as mean ±S.D.

Effect of ovariectomy on body weight

The OVX rats gained in the body weight by an average of 40 g and more weight when compared to their age and weight matched with the sham controls (Figure 5).
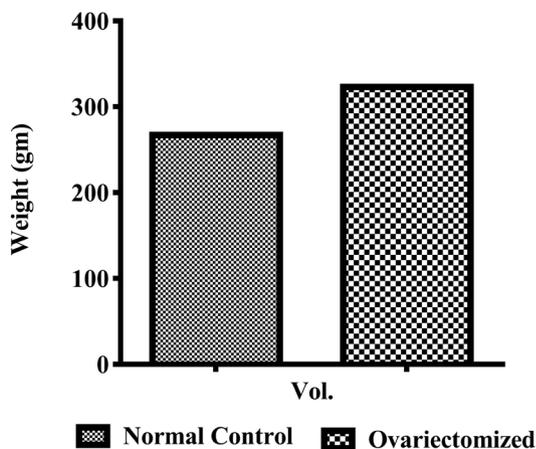

FIGURE 5 - Effect of ovariectomy on body weight.
Values are expressed as mean ±S.D.

Effect of IPTC and pharmacological intervention on myocardial infarct size

There is a raise in the myocardial infarct size significantly after promoting global ischemia for a period of 30 min and 160 min duration of reperfusion comparing with sham control. The increase in myocardial infarct size by ischemia reperfusion-induction in normal female rat heart was prevented by four cycles of 5 min of ischemia and 5 min of reperfusion but not in the OVX rat heart.

The diminished myocardial infarct size was restored after perfusion with ANP following IPTC in OVX rat

heart. While, the perfusion with L-NAME alone or combination with Glibenclamide in stabilization stage and following perfusion with ANP with four cycles of (IPTC) which weaken and diminish myocardial infarct size in the heart of OVX rat (Figure-6).
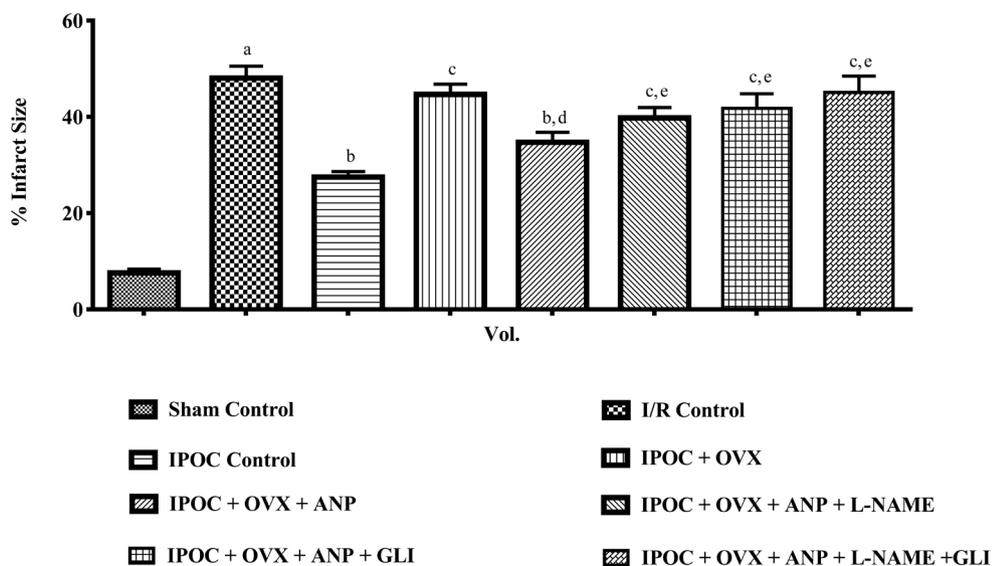

FIGURE 6 - Effect of I/R on myocardial infarct size, effect of IPTC on myocardial infarct size in normal and OVX rat heart, effect of ANP perfusion, effect of L-NAME and ANP perfusion, effect of Glibenclamide and ANP perfusion and effect of L-NAME, Glibenclamide and ANP perfusion on myocardial infarct size in heart of OVX rat.

I/R denotes ischemic reperfusion; IPTC denotes ischemic postconditioning; OVX denotes ovariectomy; ANP denotes atrial natriuretic peptide; L-NAME denotes N- nitro L-arginine methyl ester.

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. IPTC in normal rat heart; d = $p<0.05$ vs. IPTC in OVX rat heart; e = $p<0.05$ vs. IPTC in OVX rat heart with perfusion of ANP.

Effect of IPTC and pharmacological intervention on myocardial histological changes

The histological changes were observed various photomicrographs of rat heart following various groups under study (Figure-7). The photomicrograph of sham control group showed that endocardium, myocardium, epicardium, papillary muscles and vasculature were found to be normal. The myositis /necrosis were not seen under microscope (Figure 7a). The I/R control treated group was confirming lymphocytic infiltration and focal myo-necrosis with myophagocytosis. The prominent oedema following chronic inflammatory cells is visible in sub-endocardium, under microscope (Figure 7b).

IPTC treated group is showing lesser infiltration of inflammatory cells and decreased degree of myonecrosis. While, myophagocytosis and sub-endocardium vacuolar changes are seen under microscope (Figure 7c). IPTC+OVX treated group showed some severity of myonecrosis whereas less infiltration of inflammatory cells as well as a decrease in myophagocytosis when compared to IPTC. While, subendocardium vacuolar changes were also seen under microscope (Figure 7d).IPTC+OVX+ANP treated group was illustrating less severity of myonecrosis, infiltration in lymph vessels and myophagocytosis. Very little infiltration and oedema of inflammatory cells which were clearly seen under microscope (Figure 7e). IPTC+OVX+ANP+L-NAME treated group was illustrating higher degree of myonecrosis, infiltration in lymph vessels and myophagocytosis. Very little infiltration and oedema of inflammatory cells were showed observed when compared to IPTC+OVX+ANP

treated group (Figure 7f). IPTC+OVX+ANP+GLI and IPTC+OVX+ANP+L-NAME+GLI treated groups showed minute changes comparing to IPTC+OVX+ANP treated group in which there was no prominent regenerative changes seen under microscope. (Figure 7g and 7h).
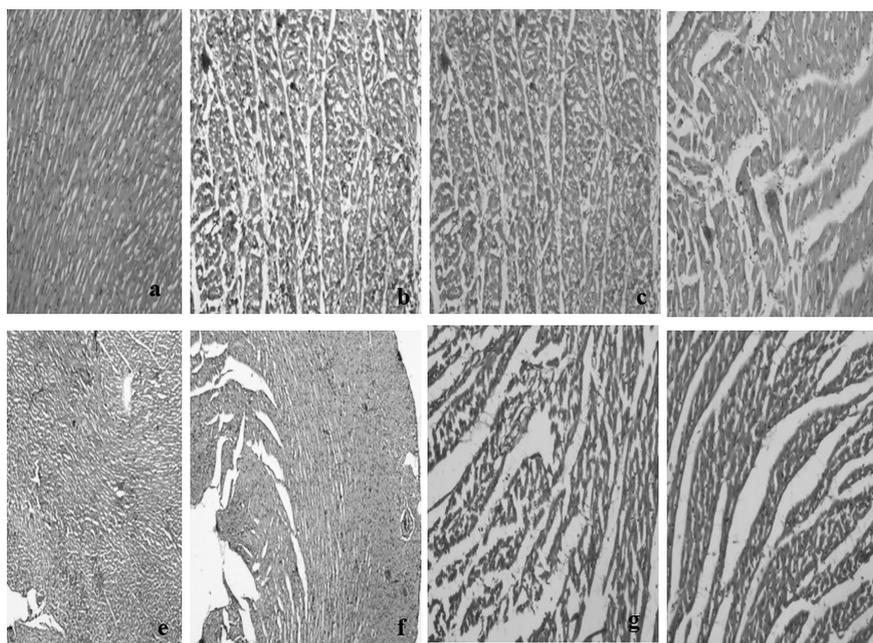

FIGURE 7 - Effect of I/R on histopathological report of heart, effect of IPTC on histopathological change in normal and OVX rat heart, effect of ANP perfusion, effect of L-NAME and ANP perfusion, effect of Glibenclamide and ANP perfusion and effect of L-NAME, Glibenclamide and ANP perfusion on histopathological change in OVX rat heart. In the Figure 7a:Sham control rat heart shows the normal cytoarchitecture of the myocardium; 7b: I/R control treated rat heart shows the necrotic changes in myocardial tissue; 7c: IPTC treated rat heart shows regenerative changes in myocardial tissue; 7d: IPTC + OVX treated rat heart shows less regenerative changes in myocardial tissue as compare to IPTC treated rat heart; 7e: IPTC + OVX + ANP treated rat heart shows more regenerative changes in myocardial tissue as compare to IPTC + OVX treated rat heart; 7f: IPTC + OVX + ANP + L-NAME treated rat heart shows less regenerative changes in myocardial tissue as compare to IPTC + OVX + ANP treated rat heart; 7g: IPTC + OVX + ANP + GLI treated rat heart shows comparative same as group IPTC + OVX + ANP + L-NAME; 7h: IPTC + OVX + ANP + L-NAME+ GLI treated rat heart shows comparative same as group IPTC + OVX + ANP + L-NAME.

I/R denotes ischemic reperfusion; IPTC denotes ischemic postconditioning; OVX denotes ovariectomy; ANP natriuretic peptide; L-NAME denotes N- nitro L-arginine methyl ester, GLI denotes Glibenclamide.

Effect of IPTC and pharmacological intervention on release of CK-MB and LDH

A discharge of CK-MB and LDH become increased significantly when a group giving global ischemia for duration of 30 min following reperfusion of 160 min comparing with sham control. The four cycles of 5 min duration of ischemia following 5 min of reperfusion were adequate to clearly prevent this release but not in OVX rat heart.

The ischemia reperfusion induces the release of CK-MB and LDH in normal female rat heart while, diminish the release of LDH and CK-MB after giving IPTC which was conspicuously restored with perfusion of ANP in OVX rat heart. Besides, perfusion of ANP in four cycles of IPTC which weaken and diminish the release of CK-MB and LDH in the heart of OVX rat after the perfusion with L-NAME alone or combination with Glibenclamide for stabilization (Figure 8 and 9).
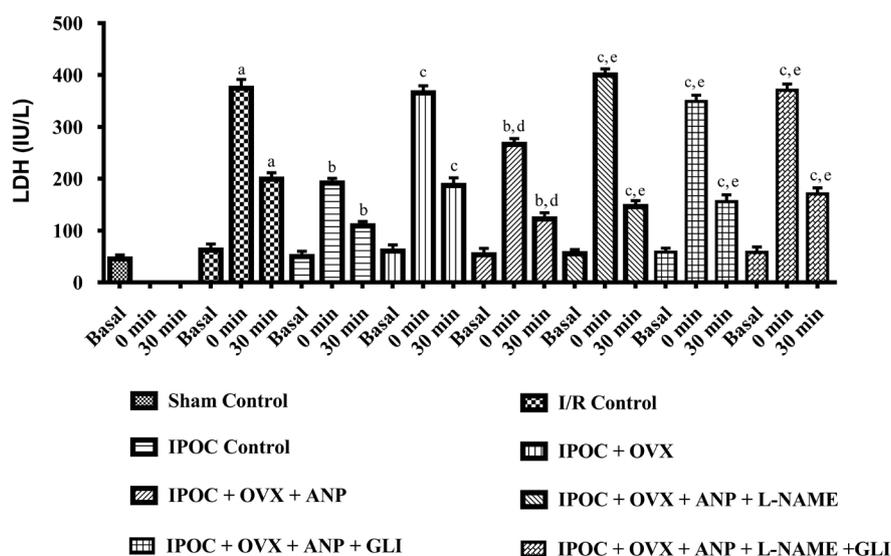
FIGURE 8 - Effect of I/R on release of LDH, effect of IPTC on release of LDH in normal and OVX rat heart, effect of ANP perfusion, effect of L-NAME and ANP perfusion, effect of Glibenclamide and ANP perfusion and effect of L-NAME, Glibenclamide and ANP perfusion on release of LDH in OVX rat heart.

I/R denotes ischemicreperfusion; IPTC denotes ischemic postconditioning; OVX denotes ovariectomy; ANP denotesatrial natriuretic peptide; L-NAME denotes N- nitro L-arginine methyl ester.

Values are expressed as mean ±S.D, a = $p<0.05$ vs. sham control; b = $p<0.05$ vs. 1 I/R control; c = $p<0.05$ vs. IPTC in normal rat heart; d = $p<0.05$ vs. IPTC in OVX rat heart; e = $p<0.05$ vs. IPTC in OVX rat heart with perfusion of ANP.
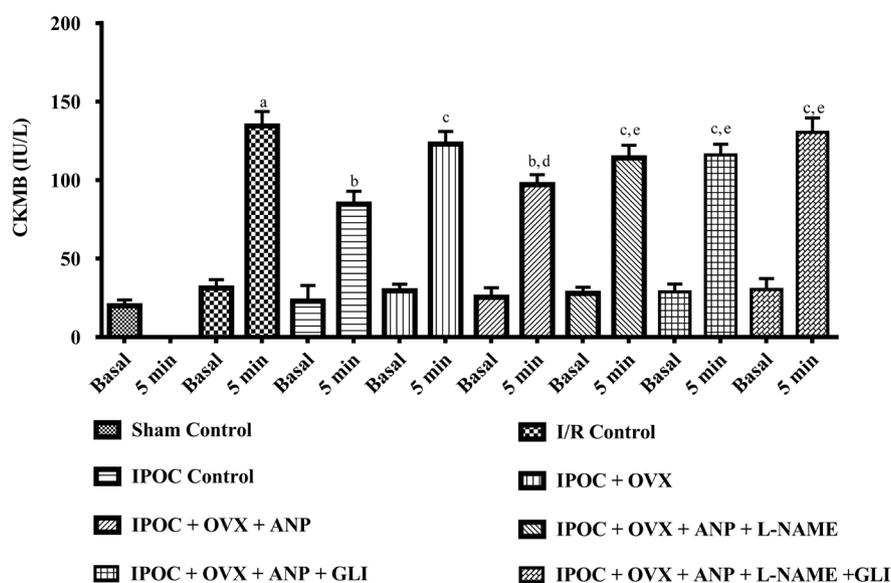

FIGURE 9 - Effect of I/R on release of CK-MB, effect of IPTC on release of CK-MB in normal and OVX rat heart, effect of ANP perfusion, effect of L-NAME and ANP perfusion, effect of Glibenclamide and ANP perfusion and effect of L-NAME, Glibenclamide and ANP perfusion on release of CK-MB in OVX rat heart.

I/R denotes ischemic reperfusion; IPTC denotes ischemic postconditioning; OVX denotes ovariectomy; ANP denotes atrial natriuretic peptide; L-NAME denotes N- nitro L-arginine methyl ester.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. IPTC in normal rat heart; d = $p<0.05$ vs. IPTC in OVX rat heart; e = $p<0.05$vs. IPTC in OVX rat heart with perfusion of ANP.

Effect of IPTC and pharmacological intervention on release of nitrite

Four events of IPTC in normal animals altogether increase nitrite release into coronary effluent when compared to I/R group but not in OVX rat heart.

An increase in the nitrite release in OVX rat heart following IPTC was observed after treatment with ANP (0.1µM/L). However, perfusion with L-NAME alone or combination with Glibenclamide in stabilization stage declined the increase in nitrite release in further OVX rat heart by perfusion with ANP (Figure 10).
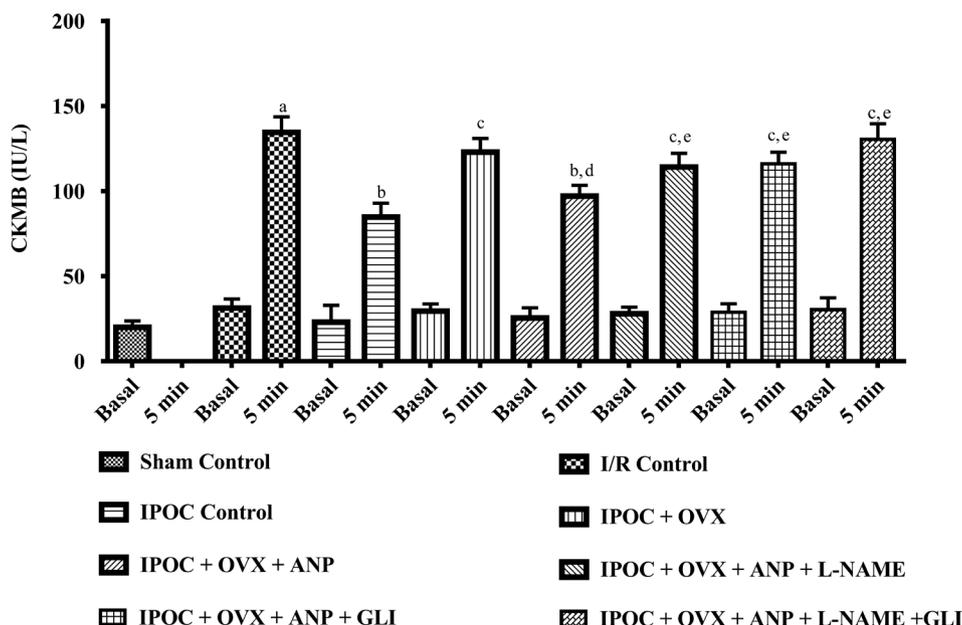

FIGURE 10 - Effect of I/R on release of nitrite, effect of IPTC on release of nitrite in normal and OVX rat heart, effect of ANP perfusion, effect of L-NAME and ANP perfusion, effect of Glibenclamide and ANP perfusion and effect of L-NAME, Glibenclamide and ANP perfusion on release of nitrite in OVX rat heart.

I/R denotes ischemicreperfusion; IPTC denotes ischemic postconditioning; OVX denotes ovariectomy; ANP denotesatrial natriuretic peptide; L-NAME denotes N- nitro L-arginine methyl ester.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. IPTC in normal rat heart; d = $p<0.05$ vs. IPTC in OVX rat heart; e = $p<0.05$vs. IPTC in OVX rat heart with perfusion of ANP.

Effect of IPTC and pharmacological intervention that ANP enhances heart rate

In this study, the heart rate is decreased in attenuation of IPTC in OVX rats at 1 min time period. ANP enhanced heart rate applying IPTC in OVX rat. While, L-NAME alone or combination with Glibenclamide significantly increased heart rate in the stabilization stage in heart of OVX rat following perfusion with ANP. This effect persisted up to 120 min of this experimentation (Figure 11).
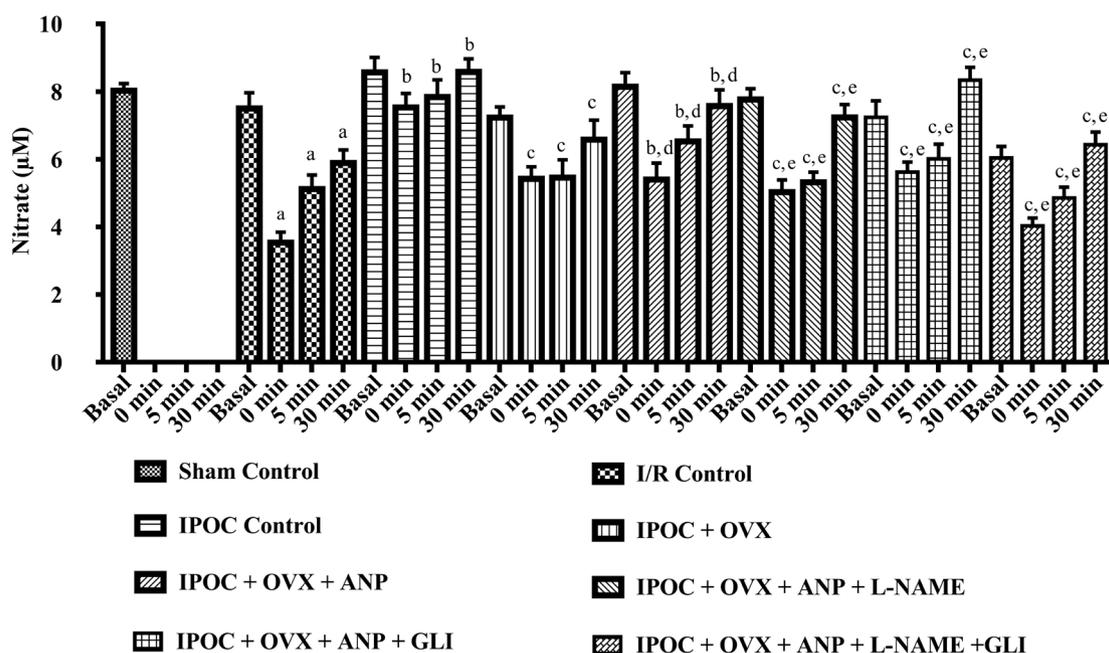
FIGURE 11 - Effect of I/R on heart rate, effect of IPTC on heart rate in normal and OVX rat heart, effect of ANP perfusion, effect of L-NAME and ANP perfusion, effect of Glibenclamide and ANP perfusion and effect of L-NAME, Glibenclamide and ANP perfusion on heart rate in OVX rat heart.

I/R denotes ischemicreperfusion; IPTC denotes ischemic postconditioning; OVX denotes ovariectomy; ANP denotesatrial natriuretic peptide; L-NAME denotes N- nitro L-arginine methyl ester.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. IPTC in normal rat heart; d = $p<0.05$ vs. IPTC in OVX rat heart; e = $p<0.05$vs. IPTC in OVX rat heart with perfusion of ANP.

DISCUSSION

The partial role of PKCε in the impaired translocation to membrane fraction and phosphorylation of PKCε and PDK1 are responsible for loss of cardioprotective effect produced by IPTC in heart of OVX rat. But, the restoration of IPTC occurs because of replacement of estrogen or selective activation of PKCε-mediated signaling. It causes translocation, phosphorylation of PKCε and phosphorylation of PDK1 (Hirschl *et al.*, 1996).

It has been reported that the protein expression of eNOS of cardiac tissue become reduced in ovariectomy condition (surgical menopause). This reduction is due to the upregulation of inhibitory protein caveolin (Liao *et al.*, 2002). The chronic treatment with estrogens restores normal activity of eNOS in myocardium. Endogenous and exogenous estrogens protect against the cardiovascular disease, in premenopausal and postmenopausal women, respectively (Pelligrino *et al.*, 2000; Shinmura *et al.*, 2008).

Apart from myocardial infarct size, LDH and CK-MB release was further weakened during four cycles of IPTC in the normal heart whereas nitrite release in the coronary effluent and heart rate were attenuated which was supported by histopathology of normal rat heart. These outcomes were found in alignment with reported papers (Ajmani *et al.*, 2011).The IPTC initiated cardio-protection in normal rat heart is explained by different mechanisms; one of which is PI-3K signaling pathway (Balakumar *et al.*, 2008).

In fact, this argument is upheld by I/R injury after regional or global ischemia which includes damages to endothelial cell, cardiomyocytes and vascular smooth muscle. The perfusion of ANP with K-H buffer in the isolated rat heart for 5 min reduces I/R reduced myocardial infract size.

The administration of ANP ensured the myocardium at the time of reperfusion from I/R injury (Sangwa et al., 2004) which controls myocardial infarct size (Yang *et al.*, 2006). It has been reported that ANP increases NO generation through activation of eNOS subsequently and limits the myocardial infarct size (De Bold, 1979).

Recently, it is reported that an endogenous cardio-protection which exerts protection as similar to IPTC (Kin *et al.*, 2004). In this study, we found that attenuation of cardio-protective effect in the heart of OVX rat with 30 min of ischemia and 120 min duration of reperfusion using IPTC due to ANP perfusion. Ultimately, this improvement lead to reduction of myocardial damage observed as reduction of infarct size, myocardial cell damage, release of CK-MB, LDH while increase in heart rate and nitrite release.

The perfusion of ANP along with L-NAME controls the IPTC induced increase in myocardial infarct size, release of CK-MB and LDH, myocardial cell damage and decrease in heart rate, release of NO in the coronary effluent of OVX rat heart. The results indicate that ANP facilitates the release of NO which protects myocardium. In present study, IPTC-induced cardio-protection and nitrite release in OVX rat heart were prominently reduced when compared with normal rat heart. It may be due to decrease in the level of ANP which cause the decrease in eNOS activity.

ANP was observed to increase eNOS expression (Fujii*et al.*, 2012) which facilitates release of NO. ANP protects myocardium from I/R injury which is further confirmed by increase in the nitrite release of a coronary effluent in OVX rat heart. However, it is well known that cardio-protection produced by remote preconditioning is due to opening of ATP sensitive potassium channels. Besides, ANP treated in OVX rat heart, the perfusion with Glibenclamide, a K_{ATP} channel blocker abolishes cardio-protective effect mediated nitric oxide release without influencing the IPTC.

The data indicates that ANP with intervention of IPTC in OVX rat heart protects the myocardium from injury. Therefore, these discoveries give us the suggestions for post-menopausal women undertaking cardiopulmonary bypass treatments. ANP reperfusion in controlled manner might be a supportive way for protection of myocardium during open heart surgery.

CONCLUSION

From the above results, it has been concluded that ANP restores the weakened action of cardio-protection together with IPTC in the heart of OVX rat. The cardio-protection has been explained by ANP mediated activation of NO pathway. ANP activated protective effect of IPTC in the heart of OVX rat is reversed by perfusion of L-NAME, an eNOS inhibitor and Glibenclamide only or in its combination and leads to decrease of myocardial infarct size. Thus, a possible alternative to sustain the cardio-protective effects of IPTC in ischemia reperfusion injury which could be broadened by ANP activation. Besides it, IPTC+ANP decrease cardiac I/R injury significantly which are further supported after getting histopathological examinations.

Post-conditioning is simple practical methodology to diminish ischemia-reperfusion injury because of small length of episode as well as duration of reperfusion which must be improved after some time of accomplishment. It has been recently suggested that methodology become widely used in the clinical aspects.

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