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Cramming the causative mechanism of glycogen synthase kinase-3β mediated by ischemic preconditioning against ovariectomy challenged rat heart

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ABSTRACT

High risks of cardiovascular diseases in women are associated with low estrogen levels. Ischemic preconditioning (IPC) exhibits protection in the heart by Glycogen synthase kinase-3β **(**GSK-3β) phosphorylation that inhibits the mPTP opening, and this protective action of IPC is attenuated by estrogen deficiency. An experiment was performed on female Wistar rats with/without ovariectomy (OVX). Isolated rat heart was attached with perfusion assembly. Infract size, coronary flow, LDH, CKMB and histopathology were estimated. Sham control group decreased the LDH, CKMB and infract size in normal rat heart. The IPC mediated protection of heart was attenuated in OVX rat heart. Inhibition of GSK-3β is found to enhance the threshold of mPTP opening during reperfusion. The treatment with atractyloside stuck significantly the protection of heart of IPC in normal and OVX rat heart. These observations show that downregulation of GSK-3β through an impaired opening of mPTP during reperfusion and GSK-3β might be potential adjuvant to IPC against cardiac injury in OVX challenged rats.

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INTRODUCTION

The clinical effects of cardiovascular studies in postmenopausal women are disappointing and inconsistent yet. After menopause, the lower level of estrogens in females increases the risk of cardiovascular diseases. It creates new studies on the actions of 17β-estradiol on the heart. It has also been reported that estrogens have a protective role in animal models associated with cardiac complications like arrhythmia, atherosclerosis (Booth *et al.*, 2008). Estrogen also reduces the episode of ischemia-reperfusion (IR) injury, myocardial infarct size, and also neutrophil infiltration in the cardiac muscles (Posa *et al.*, 2017). The normal functioning of the myocardium is notably restored by reperfusion of ischemic heart (Topol *et al.*, 1992). While sudden reperfusion of ischemic heart produced again injury of cardiac tissue termed as IR injury (Collard and Gelman, 2001). Ischemic preconditioning (IPC) is a useful protective phenomenon of heart against the short and long event of myocardial ischemia following reperfusion which also produces the protection against prolonged ischemia (Murry *et al.*, 1986). It is reported, that IPC exhibited a protec-

tive effect against IR injury by reducing myocardial infarct size, oxidative stress, accumulation of neutrophils (Sharma *et al.*, 2010).

However, cardioprotection produced by IPC gets weakened in specific pathological conditions including hypertension (Snoeckx *et al.*, 1986, 1993), aging (Abete *et al.*, 1996), heart failure (Ferdinandy *et al.*, 1998, 2007), diabetes mellitus (Ajmani *et al.*, 2011; Yadav *et al.*, 2010b) and hyperlipidemia (Yadav *et al.*, 2010a). The estrogen deficiency is a big risk factor ischemic cardiovascular disease (Booth *et al.*, 2008). Hence the accurate nature of the mechanism of attenuation of protection phenomenon in estrogen deficiency is still controversial.

The mPTP is the pore of an inner mitochondrial membrane that open on reperfusion damage mainly through Ca++ overload, oxidative stress, increased matrix pH and decrease ATP level. This opening causes decreases the mitochondrial ATP level and uncoupling of oxidative phosphorylation. Cyclosporine-A exhibit protection against I/R damage by inhibiting the mPTP opening (Yadav *et al.*, 2010b). It has been proof that both erythropoietin and IPC show protection mediated through inactivation of GSK-3β and its phosphorylation (Nishihara *et al.*, 2007).

Hence, the present study is planned to explore the role of 4-benzyl-2-methyl-1, 2, 4-thiadiazolidine-3, 5-dione (TDTZ-8) as GSK-3β inhibitors in the cardioprotection. The cardioprotection mediated through IPC would be investigated in the OVX-challenged rat heart. Finally, infract size, LDH, CKMB and coronary flow would be estimated in the various experiments to find signaling pathway (involved in cardioprotection) by low estrogen.

MATERIALS AND METHODS

Animals

In the experiment, Wistar rats (female) weight about 160-200 gm were used in this study. These experimental rats were kept in the animal cages following a cycle of light and dark (12 hours each). This research work was approved by the authorities of Institutional Animal Ethics Committee using laboratory animals.

Chemicals and drugs

TDTZ-8 and atractyloside were procured from Helix Bioscience, India. These drugs were put into the Krebs-Henseleit (KH) buffer solution for the perfusion of rat heart. All the reagents of analytical grade were freshly prepared for the experiment before use.

Induction in ovariectomy rats

Bilateral ovariectomy produced an estrogen level expressed as pg/ml, as described previously (Vishwakarma *et al.*, 2018).

Isolated rat heart preparation

The Heart was rapidly excised from heparinized rats and directly suspended to Langendorff's apparatus before starting the experiment. This isolated heart was now covered with the double-walled jacket by maintaining temperature to 37°C using hot water circulation. The heart was retrospectively perfused at a coronary flow rate of 7-9 mL/min by maintaining the pressure of 80 mmHg using KH buffer solution which comprised of composition ($MgSO_4.7H_2O$ 1.2 mM; KCl 4.7 mM; NaCl 118 mM; glucose- 11mM; KH_2PO4 1.2 mM, $NaHCO_3$ 25 mM; $CaCl_2$ 2.5 mM to get pH 7.4). The temperature was maintained to 37°C, and also passed the bubble of 5% CO_2 and 95% O2 (Hosseini *et al.*, 2020).

Experimental protocol and induction of IPC

The study was performed on nine groups of female Wistar rats, and each group contained six rats (n=6). The detailed set of groups for an experiment is shown in Figure 1 and described here,

1. Sham Control, where n = 6: Isolated heart was subjected to 10 min of stabilization and then perfused with KH buffer for 190 min continuously. At this stage, there is no global ischemia.
2. IR Control; where n = 6: After 10 min of stabilization, the isolated heart was exposed to global ischemia for 30 min followed by reperfusion with KH buffer for 120 min continuously.
3. IPC Control; where n=6: The heart was kept for 4 cycles of IPC after 10 min of stabilization. Each cycle 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.
4. IPC in OVX rat; where n = 6: Isolated OVX rat heart was kept for 4 cycles of IPC as reported earlier in group-3.
5. IPC in pretreated with TDTZ-8 (1mg/kg), surgery operated OVX rats; n = 6: Preparation of isolated pretreated OVX rat heart with TDTZ-8 (1 mg/kg dose was given, in abdominal cavity 30 min prior) OVX rat was kept for 4 cycles of IPC and rest protocol as described in group-3.
6. IPC in pretreated with TDTZ-8 (1mg/kg)and atractyloside (20 μM) perfused in surgery operated OVX rats; n = 6: Preparation of isolated

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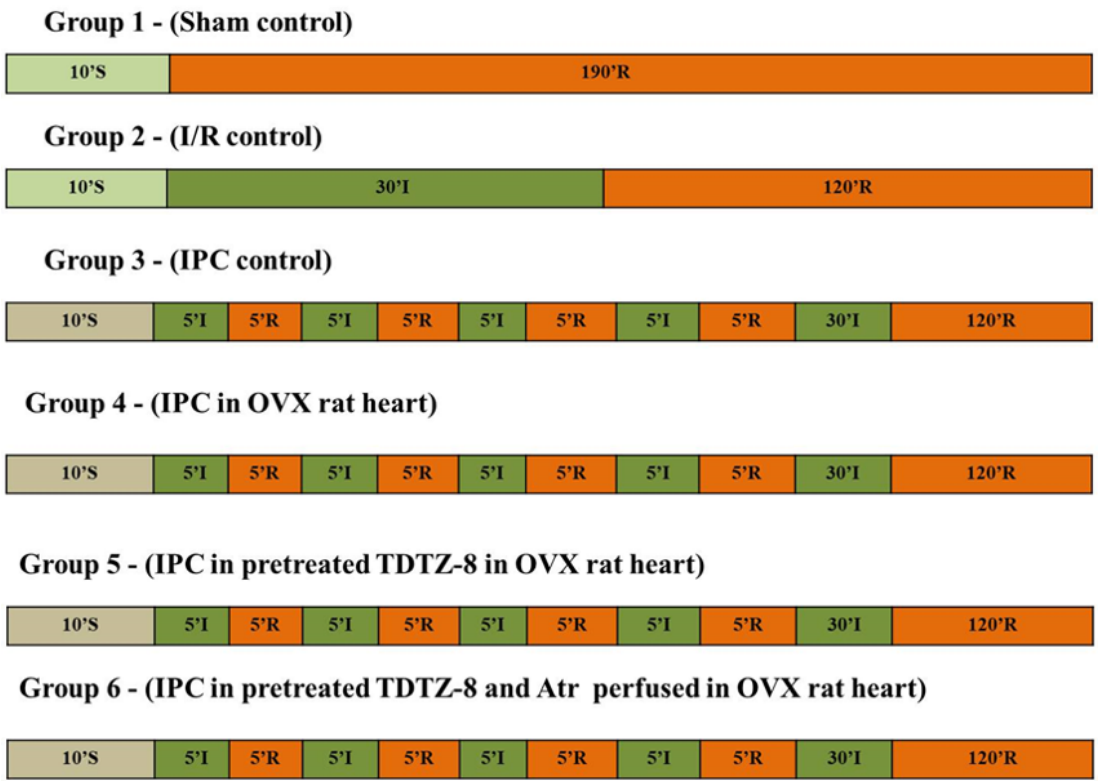

Figure 1: Diagrammatic representation of an experimental protocol

pretreated OVX rat heart with TDTZ-8 (1 mg/kg dose was given, in abdominal cavity 30 min prior) and mPTP opener drug atractyloside (20 μMKH buffer) for each episode of 5 min reperfusion and rest protocol as described in group-3.

Evaluation of myocardial infarct area

Isolated hearts were stored at −80°C for 20 to 30 min. The slices were taken after cutting the frozen heart from apex to base. Each slide was measured by a thickness of 2 to 3 mm. The triphenyl-tetrazolium chloride solution (TTC; Sigma-Aldrich, USA) was used to stain prepared slices.

The brick red color was stained for living myocardial tissues, while, the infarct area remained unstained. The per cent infarct area was measured using Image J-software in relation to a total area of the heart (NIH, Bethesda, MD, USA) (Pachauri *et al.*, 2017).

Measurement of cellular injury

In the coronary effluent from heart preparation, the LDH and CKMB levels were determined for assessing the extent of cardiac injury in experimental.

After an experiments, these levels have been estimated spectrophotometrically in the perfusate using commercial detection kits (Coral Clinical Systems Pvt. Ltd., India) (Pachauri *et al.*, 2017).

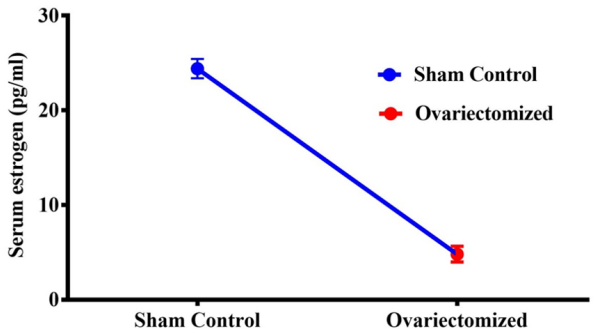

Figure 2: Effect of ovariectomyon estrogen level

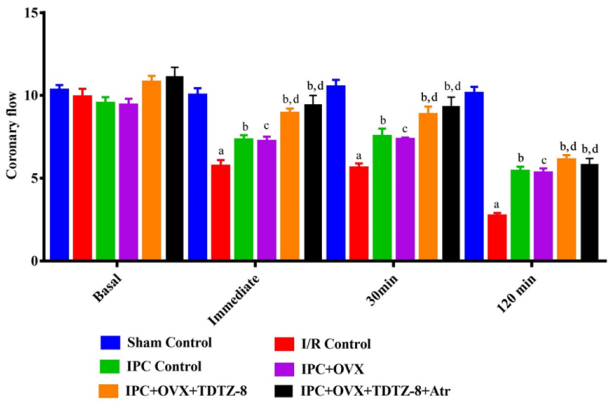

Figure 3: Effect of TDTZ-8 on the coronary flow

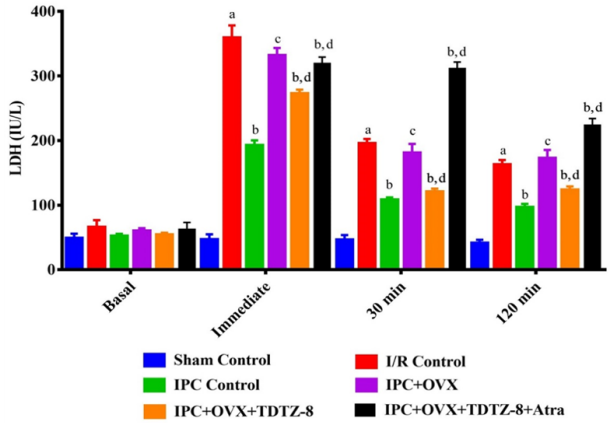

Figure 4: Effect of TDTZ-8 on the LDH

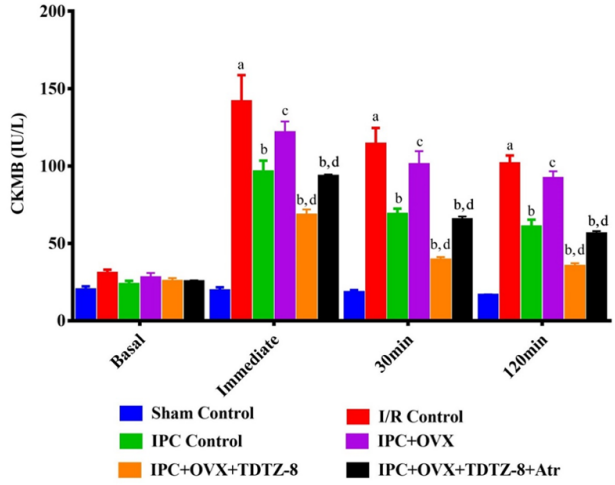

Figure 5: Effect of TDTZ-8 on the CKMB

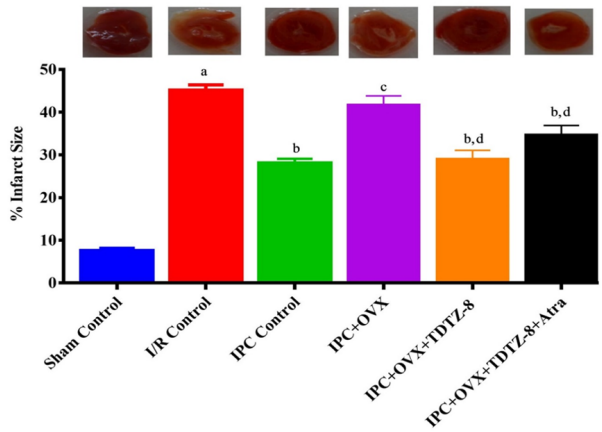

Figure 6: Effect of TDTZ-8 on the heart infarct size

Histopathological examination

Generally, 10% buffered neutral formalin solution was used for tissue fixing in the histological experiment. After fixing, these tissues were placed in paraffin-wax, and then cut transverse midventricular sections (about 5 μm thickness) using a microtome. These sections were stained using haematoxylin and eosin (Srivastav *et al.*, 2013).

Statistically analysis

All data values were expressed as mean$\pm$SD. The data of groups were statistically measured using one way ANOVA followed by Tukey's multiple comparisons test. The statistical significant value was considered as a p-value of less than 0.05.

RESULTS

Serum β-estradiol levels

The level of β-estradiol was significantly decreased in the OVX group. The level of β-estradiol did not show to zero because the ovary and adrenal gland secrete little amount female sex hormone (Figure 2).

Effect of TDTZ-8 on the coronary flow

Effect of TDTZ-8 (3 μM) on the coronary flow in OVX rat heart mediating IPC has been observed which depicted in Figure 3. There was no significant difference in coronary flow at a basal time among groups in all sets of experiments. IPC mediated decrease in the coronary flow noted in OVX challenged rat heart after 1 min to ischemia exposure while TDTZ-8 produced IPC-mediated increase in the coronary flow. Further, atractyloside was given with TDTZ-8, attenuates IPC-mediated and enhanced coronary flow in OVX challenged heart. The effect carried on up to the end of the experiment.

Effect of TDTZ-8 on LDH level

Figure 4 shows the effect of TDTZ-8 (3 μM) on IPC-mediated changes, including LDH activity in OVX-challenged rats. In this study, IPC attenuated the LDH level of coronary effluent in OVX-challenged rat heart noted at 1 min, while TDTZ-8 enhance IPC-mediated reduction in LDH level. Additionally, Atractyloside, along with TDTZ-8, diminish IPC mediated and decreased LDH level in coronary effluent in OVX challenged rat heart. The effect carried on up to the end of the experiment.

Effect of TDTZ-8 on CKMB level

Effects of TDTZ-8 (3 μM) on IPC-mediated alterations in the CKMB activity of the coronary effluent of OVX-challenged rats of the experimental design in Figure 5. In this study, IPC attenuated CKMB level of coronary effluent in OVX-challenged rat heart

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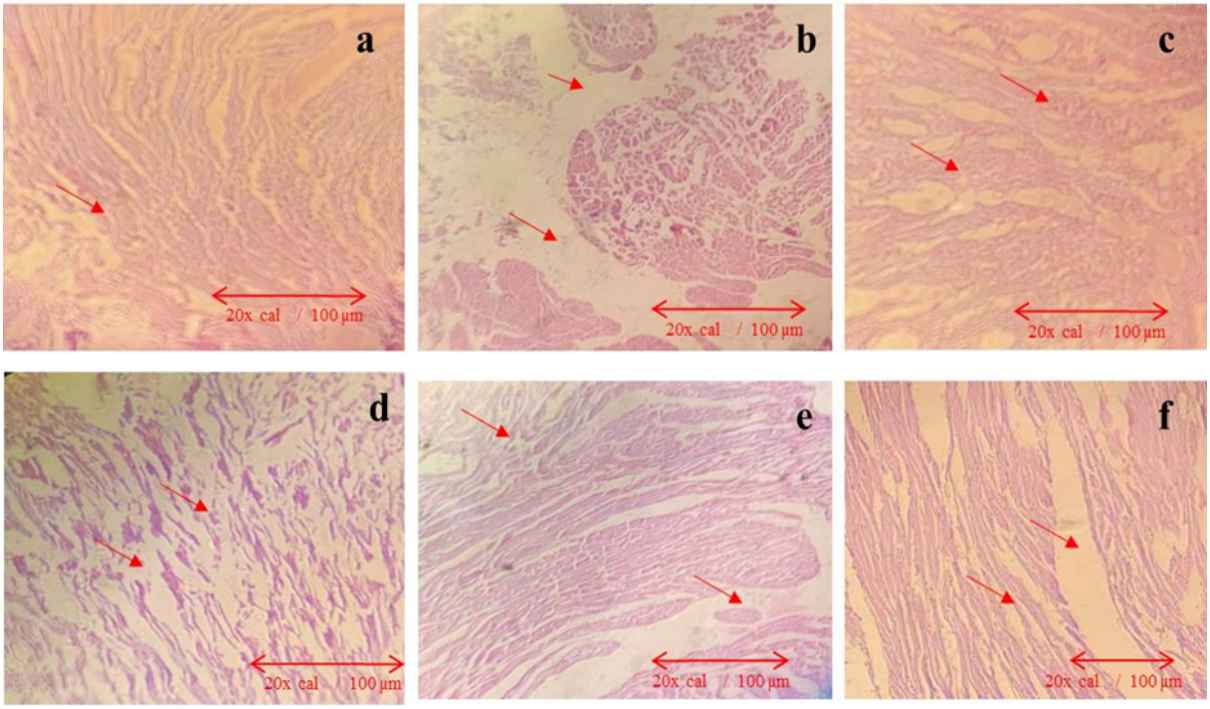

Figure 7: Effect of TDTZ-8 on histopathology

noted at 1 min, while TDTZ-8 enhance IPC-mediated reduction in CKMB level. Additionally, Atractyloside, along with TDTZ-8, diminish IPC mediated reduction in CKMB level in the coronary effluent of OVX challenged rat heart. These effects continued up to 120 min in the experiment.

Effect of TDTZ-8 on infarct size

Effect of TDTZ-8 (3 μM) on IPC-induced alterations in infarct size in OVX rats of the experimental design in Figure 6. It significantly reduces OVX rat heart infarct size in the all set of experiment. TDTZ-8 further increase the IPC mediated reduction in the infarct size in OVX rat heart. Additionally, Atractyloside, along with TDTZ-8 diminish IPC mediated decrease in infarct size in OVX heart.

Effect of TDTZ-8 on histological changes

In the Figure 7 a: Sham control rat heart shows the normal cytoarchitecture of the myocardium; Figure 7 b: IR control-treated heart shows the necrotic changes in cardiac tissue; Figure 7 c: IPC treated heart exhibit regenerative changes in cardiac tissue; Figure 7 d: IPC+OVX treated rat heart shows less regenerative changes in cardiac tissue as compared to IPC treated heart; Figure 7 e, IPC+OVX+TDTZ-8 treated heart shows more regenerative changes in cardiac tissue as compare to IPC+OVX treated rat heart; Figure 7 f, IPC+OVX+TDTZ-8+Atr treated rat heart shows less comparative same as group IPC+OVX+TDTZ-8.

DISCUSSION

In this study, four-episode of ischemia for 5 min following reperfusion of 5 min with KH buffer which was further continued to global ischemia of 30 min and reperfusion for 120 min in isolated Langendorff's perfusion with OVX heart. When it was compared with IR control group, it did not produce any significant effect. However, pretreated TDTZ-8 was given, produced significant cardioprotection against IR and IPC+OVX group. In addition, atractyloside, an mPTP opener, breaks the potentiating action of TDTZ-8 on IPC-mediated cardioprotection in OVX-challenged rats, in this research protocol. Atractyloside diminished IPC mediated cardioprotection in OVX challenged rat heart perfused with pretreated TDTZ-8 preconditioning as well as normal rat heart.

The cardiac injury was examined in terms of increased CK-MB, LDH, infarct size. The treatment with TDTZ-8 decreases the level of CK-MB, LDH enzyme in the coronary effluent and also myocardial infarct size in OVX-challenged rats. The heart was perfused with TDTZ-8 along with atractyloside restricted the decrease in the level of CK-MB, LDH enzyme and myocardial infarct size in the OVX-challenged rats.

IPC-induced cardioprotection involves many mechanisms, i.e. activation of PI3K/Akt pathway (Hausenloy and Yellon, 2007), generation of NO (Tong *et al.*, 2000), inhibition of the mPTP opening (Hausenloy, 2002) and activation of mitochon-

drial ATP-sensitive potassium channels (Oldenburg, 2002) in a normal heart. Moreover, several pharmacological interventions like an opioid receptor agonist (Gross *et al.*, 2004), an adenosine receptor agonist (Park *et al.*, 2006a), erythropoietin (Nishihara *et al.*, 2007) and bradykinin (Park *et al.*, 2006b), generate IPC like protection by consequent downregulation of GSK-3β and its phosphorylation.

The regenerative changes in myocardial tissue were attenuated by IPC in OVX-challenged rat heart. When pretreatment with TDTZ-8, it increases the effect of IPC against OVX-induced increase in histological change. Moreover, the cardioprotective effect gets attenuated in OVX rat heart when TDTZ-8 along with atractyloside. These observations support the role of GSK-3β signalling protein, which potentiates the cardioprotective effect of IPC in OVX-challenged rats. This confirms the argument of scientific data (Oldenburg, 2002) in which, GSK-3β signalling pathways inhibit mPTP opening during reperfusion.

The experimental data represented that GSK-3β inhibitors TDTZ-8 giving IPC cycle produced the cardioprotective effects on myocardium against OVX-induced cardiac injury. So, these results may have a better opportunity to treat postmenopausal females, undergoing bypass surgery. In open-heart surgery, the controlled reperfusion of pretreatment of TDTZ-8 could be a potential adjuvant for the cardioprotection.

CONCLUSIONS

The current study suggested that estrogen deficiency may cause cardiovascular risk by activating GSK-3β signalling pathway. The role of TDTZ-8 inactivates GSK-3β signaling protein through impairment of mPTP opening, which potentiates IPC mediated cardioprotective action in OVX-challenged rat heart. These signaling pathways would be used in a variety of experimental conditions associating with estrogen deficiency. Furthermore, such protective mechanisms and signaling pathways would be useful in different clinical settings in open-heart surgery and undergoing cardiopulmonary bypass surgery.

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Ethical Statement

This study was approved by the Institute Ethical Committee for Experimental Use of Animals (Permit Number: (PHAR/IAEC/18/01). The experiments were carried out in accordance with the principals and procedures of the Institute Ethical Committee for Experimental Use of Animals.

Conflict of Interest

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

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