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Remote ischemic preconditioning protects heart via modulation of purinergic signaling and AMPK-mediated autophagy in rat model of ischemia reperfusion injury

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ABSTRACT

Objective(s): Remote ischemic preconditioning (RIPC) represents a non-invasive, clinically applicable conditioning technique that mitigates myocardial ischemia-reperfusion injury (MIRI). Among various molecular mediators, the adenosine A_1 receptor (AA_1R) has emerged as a pivotal regulator of cardioprotective signaling. Currently, our aim is to elucidate the contribution of AA1R and AMPK-mediated autophagy in RIPC-induced cardioprotection.

Materials and Methods: Prolonged ischemia of 30 min and sustained reperfusion of 120 min were given to isolated hearts of rats using the Langendorff perfusion system to induce MIRI. RIPC was elicited through four intermittent phases of 5-minute limb ischemia and 5-minute reperfusion using a pressure cuff. The role of AA_1R and AMPK was investigated via pharmacological inhibition using DPCPX (selective AA_1R antagonist) and BML-275 (AMPK inhibitor), respectively.

Results: IRI-induced myocardial damage was manifested by a substantial rise in infarct size, elevated levels of cardiac-specific markers, i.e., LDH-1, CK-MB, C-tPn-I, altered hemodynamic parameters (decreased HR, CFR, LVDP, RPP, $+dp/dt_{max}$, and $-dp/dt_{min}$), and other biochemical markers (increased TBARS, decreased GSH and catalase, increased TNF-α, TGF-β, Bax, and caspase-3). RIPC significantly attenuated these deleterious alterations, restoring both biochemical and functional parameters. However, the administration of DPCPX and BML-275 markedly abrogated the cardioprotective benefits conferred by RIPC.

Conclusion: These findings substantiate that RIPC exerts potent cardioprotective effects via activation of AA_1R and AMPK-dependent autophagic signaling. The observed interplay between AA_1R and AMPK underscores an integrated adaptive mechanism that preserves myocardial integrity during IRI. This mechanistic insight provides a rationale for exploring AA1R-AMPK axis modulation as a therapeutic avenue for clinical cardioprotection.

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Introduction

Cardiovascular diseases (CVDs) are reported to be the leading cause of mortality across the world, contributing to nearly 18.6 million mortalities annually, which is almost equivalent to about 31% of total worldwide mortality (1). Among these, acute myocardial infarction (AMI) is the most prevalent condition, responsible for about 15.9 million deaths annually (2). AMI occurs due to complete obstruction of the coronary artery, which further leads to irreversible loss of cardiomyocytes (3). Restoration of blood flow is the only effective intervention to minimize ischemic injury. Although reperfusion is vital for restoring blood flow to the ischemic heart, its sudden onset may paradoxically aggravate tissue injury, a phenomenon termed myocardial ischemia–reperfusion injury (MIRI)(4). Besides pharmacological thrombolysis, several conditioning strategies have been explored to mitigate MIRI and improve post-ischemic cardiac recovery. Ischemic preconditioning and postconditioning are documented to exhibit significant myocardial protection in various pre-clinical (5, 6) and clinical (7) studies of ischemia-reperfusion injury (IRI). Among these strategies, remote ischemic preconditioning (RIPC) represents a more practical, non-invasive, and safer technique. It involves inducing brief, reversible periods of ischemia with subsequent reperfusion in an organ or tissue other than the target site, thereby triggering systemic protective mechanisms that protect the myocardium from subsequent ischemic damage (8). Several clinical studies have demonstrated the myocardium-protective potential of RIPC in reducing myocardial injury (9, 10). Multiple molecular signaling cascades, including JAK-STAT3, RAGE-HMGB1, and SDF-1α-CXCR4, have been shown to play a crucial role in cardioprotection via RIPC (11, 12). Adenosine is a purine nucleoside consisting of an adenine base linked to a ribose

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sugar through a β-N9-glycosidic bond. It plays an intricate role in regulating several physiological processes, including heart rate, myocardial contractility, and vascular tone (4,6). The intracellular concentration of adenosine markedly increases under conditions of disrupted energy homeostasis (13). Moreover, exogenous administration of adenosine has been shown to activate multiple protective metabolic pathways, thereby mitigating MIRI (14, 15). Adenosine A_1 subtypes receptor AA_1R is a type of glycoprotein; expressed on the membrane of multiple organs (predominantly heart)(16). Many research findings have documented that adenosine has an intricate role in triggering RIPC across various organs, including the limb, renal, mesenteric, and carotid arteries (8, 17).

Autophagy is an evolutionarily conserved cellular process that degrades and recycles damaged organelles and cytoplasmic proteins (18), functioning in both normal and abnormal conditions, including nutrient deficiency, ATP deprivation, ischemia, and hypoxia (19). IRI attenuation has been reported to involve modulation of various autophagic signaling pathways, either through activation or inhibition (20). Moreover, studies have evidenced an association between the beneficial effects of autophagy and activation of AA_1 receptor in multiple pathological conditions, including cardiovascular disorders (21).

AMP-activated protein kinase (AMPK), a serine/threonine kinase, acts as a master energy sensor that maintains cellular energy homeostasis by phosphorylating downstream targets involved in metabolic regulation and cell survival during energy stress (22). Preclinical studies have demonstrated that AMPK-mediated autophagy has a crucial role in mitigating IRI in multiple organs (23, 24). But the role of AMPK-mediated autophagy in the myocardial protection provided through RIPC during MIRI remains unexplored. Therefore, we designed this study to elucidate the potential of AA1 receptor in cardioprotective effects of RIPC, with possible involvement of AMPK-dependent autophagy signaling in the MIRI model of rats.

Materials and Methods

Experimental animals

Wistar Rats (males and females) weighing around 150-200 gr were used in this study; they were procured from DFSAH, LUVAS, Hisar, Haryana. Rats were acclimated in the institution's animal house under a 12-hour light/dark cycle and provided with standard chow (Ashirwad Industries, Chandigarh) and tap water *ad libitum*. The experimental animals protocol was approved by the Institutional Animal Ethics Committee (IAEC) (Reg. No. 107/Go/ReBi/S/99/CCSEA/2021-10), and all procedures complied with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Department of Animal Husbandry and Dairying, Govt. of India.

Drugs and chemicals

"Diphenyl cyclopentylxanthine (DPCPX) and BML-275 were procured from Aba Chem Scene Pvt. Ltd., USA. Various chemicals such as Dinitrophenylhydrazine (DNPH), Nicotinamide adenine dinucleotide (oxidized) (NAD^+), Nicotinamide adenine dinucleotide (reduced) (NADH), lactate, Triphenyl tetrazolium chloride (TTC), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 2,4-thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Hydrogen peroxide (H_2O_2) and Dimethyl sulfoxide (DMSO) were obtained from Loba Chemie Pvt Ltd., India. Bovine serum albumin (BSA) and Folin-Ciocalteu Reagent (FCR) were purchased from Sisco Research Laboratories Pvt. Ltd., India. All chemicals were of analytical grade and freshly prepared. Creatine kinase-MB (CK-MB), Cardiac troponin-I (C-tPn-I), Tumor necrosis factor-alpha (TNF-α), Transforming growth factor-beta (TGF-β), Bax, and Caspase-3 ELISA kits were procured from Krishgen Biosystems, Mumbai, Maharashtra, India. DPCPX and BML-275 were dissolved in 10% DMSO and used as selective inhibitors of AA1R- and AMPK-mediated autophagy, respectively. The doses of DPCPX (0.1 and 0.2 mg/kg; *IP*)(25) and BML-275 (1.5 and 3 mg/kg; *IP*)(26) were selected according to previously published reports.

Experimental model

Thiopental sodium (50 mg/kg; *IP*) was used to anesthetize the rats, and RIPC was given by tying the blood pressure cuff at the inguinal level of the single hind limb of the rat. The blood pressure cuff was inflated (up to 150 mm of Hg) for 5 min to induce ischemia and deflated for 5 min to re-perfuse the tissue, for four consecutive times to condition the tissue against IRI. Pre-treated heparinized hearts were excised and retrogradely perfused with Krebs-Henseleit solution at a constant pressure of 70 mm Hg, maintained at pH 7.4, an optimal temperature of 37 ˚C, and bubbled with 95% O_2 and 5% CO_2 in a Langendorff apparatus (27, 28). MIRI was assessed by inserting a latex balloon filled with fluid into the left ventricular portion of the heart to record left ventricular developed pressure (LVDP), $+dp/dt_{max}$, $-dp/dt_{min}$, and heart rate using a pressure transducer (AD Instruments, Australia). Coronary flow rate was measured at different time intervals, i.e., basal, 5, 30, 60, and 120 min after reperfusion (AR) to assess the extent of cardiac injury.

Estimation of heart infarct size

Following the reperfusion period, the heart was detached from the perfusion system and preserved for a night at 0 ˚C for further analysis. The frozen heart was then sectioned into uniform slices, each approximately 2-3 mm thick. A 1% solution of TTC was prepared in 0.2 M Tris buffer (pH 7.4) and was used to incubate these sections for 20 min at 37 ˚C. Regions of the viable myocardium were stained red; on the contrary, the infarcted, non-viable tissue did not retain the dye and appeared pale yellow in color. The infarct size was subsequently determined using 1% TTC staining, as described in a previously established method (29), employing the area-based approach (30).

Assessment of biochemical parameters

Coronary effluent collection

To assess LDH-1 and CK-MB activity, coronary effluent was collected during the stabilization (basal) phase and at 5, 30, 60, and 120 min after AR.

Supernatants collection from homogenate of heart

Following IR procedure completion, each heart was excised and weighed. The tissue was then homogenized in phosphate-buffered saline (PBS), pH 7.4. The resulting homogenate was centrifuged at 3500 rpm for 15 min at 4 ˚C, and the clear supernatant was collected and stored at -20 ˚C for subsequent biochemical estimations. Assessments

of various parameters, including C-tPn-I, total protein content, TBARS, GSH, catalase activity, TNF-α, TGF-β, Bax, and caspase-3, were conducted.

Assessment of activity of LDH-I enzyme

Estimation of LDH-1 activity was carried out in samples of the coronary effluent obtained at designated time intervals by the 2,4-dinitrophenylhydrazine (2,4-DNPH) method as discussed by King (31).

Assessment of activity of CK-MB enzyme

CK-MB activity was estimated in samples of coronary effluent collected at defined time intervals, following the standardized protocol for the marketed ELISA kit from Krishgen Biosystems, Mumbai. The CK-MB enzyme activity was estimated by taking the final absorbance at 450 nm using an ELISA reader (32).

C-tPn-I protein content measurement

Measurement of C-tPn-I protein was performed in the heart homogenate using the standard protocol of the marketed ELISA kit purchased from Krishgen Biosystems, Mumbai. The C-tPn-I protein content was assessed by measuring the final absorbance at 450 nm using an ELISA reader (33).

Heart total protein content assessment

The total protein content of the whole heart was estimated using the spectrophotometric method (Shimadzu 1800, Japan) at 750 nm, following the Lowry *et al.* (34) method.

Heart TBARS levels measurement

The level of malondialdehyde (MDA) was measured in heart homogenates using a spectrophotometer (Shimadzu 1800, Japan) at 532 nm to assess oxidative stress (35).

Heart GSH levels assessment

The Ellman method was used to spectrophotometrically (Shimadzu 1800, Japan) measure GSH content in heart homogenate at 412 nm, reflecting oxidative stress (36).

Heart catalase activity estimation

Catalase activity was quantified in the heart homogenate using the spectrophotometric method (Shimadzu 1800, Japan) at 570 nm, following Sinha's method (37) with slight modifications (38).

Heart TNF-α, TGF-β, Bax, and Caspase-3 content measurement

TNF-α, TGF-β, Bax, and Caspase-3 contents were quantified in heart homogenate using a standardized protocol with a commercially available ELISA kit from Krishgen Biosystems, Mumbai. The final absorbance was taken at 450 nm by using an ELISA reader to estimate the content of TNF-α, TGF-β, Bax, and Caspase-3 (39-41).

Experimental protocol

Presently, a total of 66 rats were used and were grouped into 11 different groups of 6 animals each.

Group I- normal control

The isolated heart of a rat was mounted on a Langendorff apparatus and perfused with KH solution for 170 min.

Group II- vehicle control

After intraperitoneal administration of 10% DMSO, the hearts were isolated and stabilized for 20 min, then perfused for 150 min with KH buffer in a Langendorff perfusion apparatus.

Group III- RIPC sham control

In anesthetized rats, a blood pressure cuff was positioned on the hind limb but not inflated. After 40 minutes, the heart was isolated and perfused using the Langendorff system. Once stabilized for 20 minutes, global ischemia was induced for 30 minutes, followed by 120 min of reperfusion.

Group IV- IRI control

After stabilization, 30 min of global ischemia followed by 120 min of reperfusion were given to isolated hearts.

Group V- RIPC control

RIPC was performed by applying four phases of 5-minute ischemia and 5-minute reperfusion to the hind limb using a pressure cuff. Subsequently, the hearts were isolated, stabilized, and exposed to prolonged ischemia of 30 min followed by 120 min of reperfusion with KH buffer.

Group VI- DPCPX (LD)+RIPC

Rats were heparinized, followed by administration of the AA1R-selective antagonist, DPCPX (0.1 mg/kg, *IP*). After 30 min, animals were anaesthetized by using thiopental sodium (50 mg/kg; *IP*), and the remaining procedure was the same as described in group V.

Group VII- DPCPX (HD)+RIPC

After heparinization, rats were administered the AA1R-selective antagonist DPCPX (0.2 mg/kg, *IP*). After 30 min, animals were anaesthetized with thiopental sodium (50 mg/kg, IP), and the remainder of the procedure was the same as described in group V.

Group VIII- BML-275 (LD)+RIPC

Rats were heparinized, followed by administration of a selective AMPK inhibitor, i.e., BML-275 (1.5 mg/kg; *IP*). After 30 min, animals were anaesthetized and then underwent the same procedure as in group V.

Group IX- BML-275 (HD)+RIPC

After heparinization, rats were subjected to the administration of a selective AMPK inhibitor, i.e., BML-275 (3 mg/kg; *IP*). After 30 min, animals were anaesthetized, and the same procedure was repeated as described in group V.

Group X- DPCPX Per se

DPCPX (0.2 mg/kg; IP) was administered, and the hearts were then isolated. After cannulation, the heart was stabilized and perfused for 150 min with KH solution on the Langendorff perfusion apparatus.

Group XI- BML-275 per se

BML-275 (3 mg/kg; IP) was injected, and the hearts were then isolated. Following stabilization, the isolated hearts were perfused for 150 min with KH solution on the Langendorff perfusion apparatus.

Statistical analysis

"Statistical analysis was performed using GraphPad Prism version 9.5.1 (733). Data were expressed as mean±SD (n=6). One-way ANOVA was used to analyze infarct size, C-tPn-I, TNF-α, TGF-β, Bax, caspase-3, TBARS, GSH, and catalase. In contrast, two-way ANOVA was applied for LDH-1, CK-MB, and hemodynamic variables (HR, LVDP, CFR, RPP, $+dP/dt_{max}$, $-dP/dt_{min}$). Bonferroni's and Tukey's *post hoc* tests were performed for multiple group comparisons. Statistical significance was set at $P<0.05$.

Results

Effects of various interventions on hemodynamic indices

Sustained ischemia of 30 min with subsequent reperfusion of 120 min substantially decreased the hemodynamic indices, including HR, LVDP, CFR, RPP, $+dp/dt_{max}$, and $-dp/dt_{min}$ in animals of the IRI control group in comparison to their basal values and the values of the normal and vehicle control group animals observed at different designated time intervals. On the other side, RIPC showed a significant reversal in all aforementioned hemodynamic parameters in comparison to the animals of the IRI control group. By contrast, no significant improvements were observed in the RIPC sham control group compared with the IRI control group. However, the beneficial effects of RIPC were markedly attenuated by pre-administration of DPCPX (an A1R subtype-selective antagonist; 0.1 and 0.2 mg/kg, *IP*). Moreover, pre-treatment of BML-275 (an AMPK-dependent autophagy inhibitor; 1.5 and 3 mg/kg; *IP*) also significantly abrogated the protective effects of RIPC (Tables 1-5).

Table 1. Effects of different interventions on rat heart rate (HR)(Beats/min)

Groups	Basal	5AR	30AR	60AR	120AR
Normal Control	286.14±21.86	289.38±25.83	283.06±17.23	295.70±19.21	279.88±9.30
Vehicle Control	291.93±28.93	291.60±20.90	290.05±17.70	301.05±19.5	274.42±13.90
RIPC Sham Control	292.55±20.49	114.81±12.58 ab#	133.66±11.12 ab$	150.18±8.5 ab$	134.54±10.50 ab*
IRI Control	284.22±22.93	110.15±15.69 ab#	125.73±14.[illegible]	142.68±12.85 ab$	125.92±12.65 ab*
RIPC Control	287.61±16.31	190.54±15.96 cd*	204.48±12.[illegible] cd	235.29±11.41 cd$	196.38±14.09 cd*
DPCPX (LD)+RIPC	296.98±21.50	137.13±11.03 abe#*	155.18±9.43 abe	175.09±13.02 abe$*	158.80±11.50 ab$*
DPCPX (HD)+RIPC	287.33±28.38	120.39±8.81 abe#*	137.69±11.49 abe#*	161.79±10.47 abe$*	158.19±8.25 ab$*
BML-275 (LD)+RIPC	297.54±25.98	125.21±12.61 abe#*	141.10±9.40 abe#*	177.86±14.36 abe$*	159.80±8.89 ab$*
BML-275 (HD)+RIPC	290.03±16.57	133.90±6.[illegible] abe#	149.20±11.91 abe#*	176.33±12.14 abe$*	153.09±14.22 ab$*
DPCPX (HD) *per se*	289.86±21.60	297.17±5.96 cd	296.09±17.03 cd	297.78±16.88 cd	271.29±12.44 cd
BML-275 (HD) *per se*	277.74±25.51	298.9[illegible]±19.3[illegible] cd	298.51±10.58 cd	295.57±19.51 cd	270.82±10.37 cd

Final values are presented as mean±SD (95% Confidence Interval) (n=6).
a= vs Normal Control; b= vs Vehicle Control; c= IRI Control; d= RIPC Sham Control; e= RIPC Control
*=$P<0.05$; $=$P<0.01$; #=$P<0.001$
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

Table 2. Effects of different interventions on rat left ventricular developed pressure (LVDP)(mmHg)

Groups	Basal	5AR	30AR	60AR	120AR
Normal Control	77.05±5.57	79.23±3.66	76.14±4.41	79.36±3.14	74.79±4.54
Vehicle Control	78.23±3.71	80.22±3.38	79.66±4.10	80.99±3.11	72.71±2.51
RIPC Sham Control	75.68±4.32	32.40±4.65 ab#	42.76±2.64 ab$	45.65±3.17 ab$	42.50±2.54 ab*
IRI Control	78.49±3.85	28.30±2.01 ab#	39.40±3.99 ab#	43.17±5.28 ab#	39.84±3.23 ab$
RIPC Control	78.56±5.06	53.44±3.02 cd$	60.01±1.91 cd*	65.21±4.00 cd$	56.92±3.83 cd*
DPCPX (LD)+RIPC	79.72±3.70	39.68±4.17 abe#*	45.86±2.90 abe#*	50.13±5.62 abe$*	47.56±3.78 ab$*
DPCPX (HD)+RIPC	81.19±3.00	34.79±3.53 abe#$	44.47±2.62 abe#*	50.11±4.00 abe#*	45.19±2.76 abe#*
BML-275 (LD)+RIPC	79.14±4.54	38.02±2.52 abe#*	47.68±2.28 abe#*	52.57±3.62 abe#*	48.60±4.91 ab$*
BML-275 (HD)+RIPC	79.58±3.94	37.28±3.70 abe#*	45.31±2.21 abe#*	51.05±3.54 abe#*	45.99±3.53 abe$*
DPCPX (HD) *per se*	76.54±4.02	78.84±3.98 cd	79.43±3.21 cd	79.10±3.06 cd	74.65±3.04 cd
BML-275 (HD) *per se*	79.58±3.49	76.28±4.16 cd	81.12±3.94 cd	79.52±3.30 cd	72.11±2.17 cd

Final values are represented as mean±SD (95% Confidence Interval)
a= vs Normal Control; b= vs Vehicle Control; c= IRI Control; d= RIPC Sham Control; e= RIPC Control
* = $P<0.05$; $ = $P<0.01$; # = $P<0.001$
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

Table 3. Effects of different interventions on rat coronary flow rate (ml/min)

Groups	Basal	5AR	30AR	60AR	120AR
Normal Control	12.95±0.51	13.13 ±0.36	13.01±0.47	13.09±0.23	12.27±0.34
Vehicle Control	12.97±0.44	13.06 ±0.55	12.80±0.49	12.96±0.18	12.12±0.32
RIPC Sham Control	13.05±0.47	6.39±0.63 $^{ab\#}$	7.10±0.46 $^{ab\#}$	7.36±0.28$^{ab\$}$	6.88±0.22 ^{ab*}
IRI Control	12.48±0.47	5.96±0.26 $^{ab\#}$	6.80±0.16 $^{ab\#}$	7.07±0.20$^{ab\#}$	6.59±0.28 $^{ab\$}$
RIPC Control	12.15±0.70	9.98±0.22 $^{cd\$*}$	10.78±0.31 $^{cd\$*}$	11.46±0.73$^{cd\$*}$	10.03±0.31 ^{cd*}
DPCPX (LD)+RIPC	12.61±0.77	8.03±0.26 $^{abe\$*}$	8.19±0.21 $^{abe\$*}$	8.92±0.48$^{abe\$*}$	7.12±0.30 $^{abe\$*}$
DPCPX (HD)+RIPC	12.78±0.52	7.82±0.32 $^{abe\#*}$	8.26±0.30 $^{abe\#*}$	8.69±0.43$^{abe\$*}$	6.95±0.39 $^{abe\$*}$
BML-275 (LD)+RIPC	12.60±0.79	8.05±0.32 $^{abe\$*}$	8.35±0.30 $^{abe\$*}$	9.07±0.44$^{abe\$*}$	7.18±0.47 $^{abe\$*}$
BML-275 (HD)+RIPC	12.53±0.66	7.91±0.51 $^{abe\#*}$	8.10±0.07 $^{abe\#*}$	9.10±0.40$^{abe\$*}$	6.98±0.26 $^{abe\$*}$
DPCPX (HD) *per se*	12.90±0.59	12.86±0.26 ^{cd}	12.92±0.28 ^{cd}	12.80±0.30^{cd}	12.85±0.41 ^{cd}
BML-275 (HD) *per se*	13.00±0.53	12.96±0.13 ^{cd}	13.04±0.27 ^{cd}	12.92±0.49^{cd}	12.31±0.23 ^{cd}

Final values are represented as mean±SD (95% Confidence Interval)
a= vs Normal Control; b= vs Vehicle Control; c= IRI Control; d= RIPC Sham Control; e= RIPC Control
* = P<0.05; $ = P<0.01; # = P<0.001
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

Table 4. Effects of different interventions on rat RPP (beats.mmHg/min)

Groups	Basal	5AR	30AR	60AR	120AR
Normal Control	22140.40±3044.26	22921.75±2225.75	21597.51±2273.70	23467.50±17[illegible]98	20930.08±1429.60
Vehicle Control	22811.04±2702.35	23399.25±1993.64	23113.19±1916.12	2437[illegible].11±16[illegible]7.20	19930.95±781.36
RIPC Sham Control	22184.00±2403.01	3676.59±367.91 $^{ab\#}$	5723.85±662.51 $^{ab\#}$	[illegible]855.56±[illegible]03.86 $^{ab\$}$	5726.57±651.12 $^{ab\$}$
IRI Control	22300.42±2065.38	3134.60±566.29 $^{ab\#}$	4909.26±3[illegible]1 $^{ab\#}$	612[illegible]9±669.59 $^{ab\#}$	5028.86±706.70 $^{ab\$}$
RIPC Control	22570.77±1675.14	10171.55±891.95 $^{cd\$*}$	12282.64±1002.90 [illegible]	15298.94±327.58 $^{cd\#\$}$	11208.71±1362.95 $^{cd\$*}$
DPCPX (LD)+RIPC	23636.26±1541.69	5418.17±522.86 $^{abe\#*}$	6647.9[illegible]472.03 $^{abe\#*}$	8744.98±910.39 $^{abe\$*}$	7516.13±336.21 $^{abe\$*}$
DPCPX (HD)+RIPC	23331.12±2460.34	4208.59±672.87 $^{abe\#*}$	610[illegible]53±39[illegible] $^{abe\#(}$	8096.41±735.56 $^{abe\#*}$	7168.95±794.72 $^{abe\$*}$
BML-275 (LD)+RIPC	23662.06±3361.16	4755.11±532.80 $^{abe\#*}$	7[illegible]16.10[illegible]20.36 $^{abe\#*}$	9333.42±825.35 $^{abe\$*}$	7726.99±467.17 $^{abe\$*}$
BML-275 (HD)+RIPC	23019.50±517.10	4988.72±520.81	6577[illegible]4±595.02 $^{abe\#*}$	9024.97±1103.25 $^{abe\$*}$	7022.35±646.9 $^{abe\$*}$
DPCPX (HD) *per se*	22162.69±1810.01	23479.75±23[illegible]4 ^{cd}	23526.37±1790.40 ^{cd}	23565.86±1790.46 ^{cd}	20250.39±1246.99 ^{cd}
BML-275 (HD) *per se*	22105.49±2344.28	22812.43±2[illegible]8.25 ^{cd}	24203.84±1254.55 ^{cd}	23506.20±1872.78 ^{cd}	19521.66±767.67 ^{cd}

Final values are represented as mean±SD (95% Confidence Interval)
a= vs Normal Control; b= vs Vehicle Control; c= IRI Control; d= [illegible]C Sham Control; e= RIPC Control
* = P<0.05; $ = P<0.01; # = P<0.001
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfu[illegible]on injury; DPCPX: Diphenyl cyclopentylxanthine

Table 5. Effects of different interventions on +dp/dt$_{max}$ and -dp/dt$_{min}$ (mmHg/sec/sec) in rats

Groups	+dp/dt$_{max}$ (mmHg/sec/sec)		-dp/dt$_{min}$ (mmHg/sec/sec)	
	Basal	120AR	Basal	120AR
Normal Control	6675.93±138.85	6585.12 ±167.86	-6724.29±132.63	-6658.01±138.69
Vehicle Control	6813.72±117.90	6510.50 ±166.08	-6804.47±202.15	-6478.48±131.50
RIPC Sham Control	6866.43±208.01	2209.63 ±97.86 $^{ab\#}$	-6736.76±113.74	-1994.94±98.35 $^{ab\#}$
IRI Control	6837.69±178.56	2036.59 ±76.35 $^{ab\#}$	-6961.71±129.07	-1950.19±127.88 $^{ab\#}$
RIPC Control	6598.55±148.93	4660.69 ±90.88 $^{cd\$*}$	-6633.26±180.52	-4372.20±130.71 $^{cd\$*}$
DPCPX (LD)+RIPC	6735.70±176.86	2832.99 ±134.07 $^{abe\#*}$	-6754.02±253.25	-2821.37±109.11 $^{abe\#*}$
DPCPX (HD)+RIPC	6848.52±215.82	2706.94 ±92.06 $^{abe\#*}$	-6870.81±302.23	-2772.61±110.33 $^{abe\#*}$
BML-275 (LD)+RIPC	6761.78±178.29	2869.29±108.56 $^{abe\#*}$	-6637.80±249.08	-2794.49±99.27 $^{abe\#*}$
BML-275 (HD)+RIPC	6790.25±100.57	2778.05±88.15 $^{abe\#*}$	-6943.59±136.07	-2713.59±151.54 $^{abe\#*}$
DPCPX (HD) *per se*	7158.12±152.67	6724.19±200.85 ^{cd}	-6787.12±220.91	-6611.11±201.46 ^{cd}
BML-275 (HD) *per se*	6808.75±141.25	6814.12±86.15 ^{cd}	-6868.43±117.31	-6582.93±176.80 ^{cd}

Final values are represented as mean±SD (95% Confidence Interval)
a= vs Normal Control; b= vs Vehicle Control; c= IRI Control; d= RIPC Sham Control; e= RIPC Control
* = P<0.05; $ = P<0.01; # = P<0.001
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

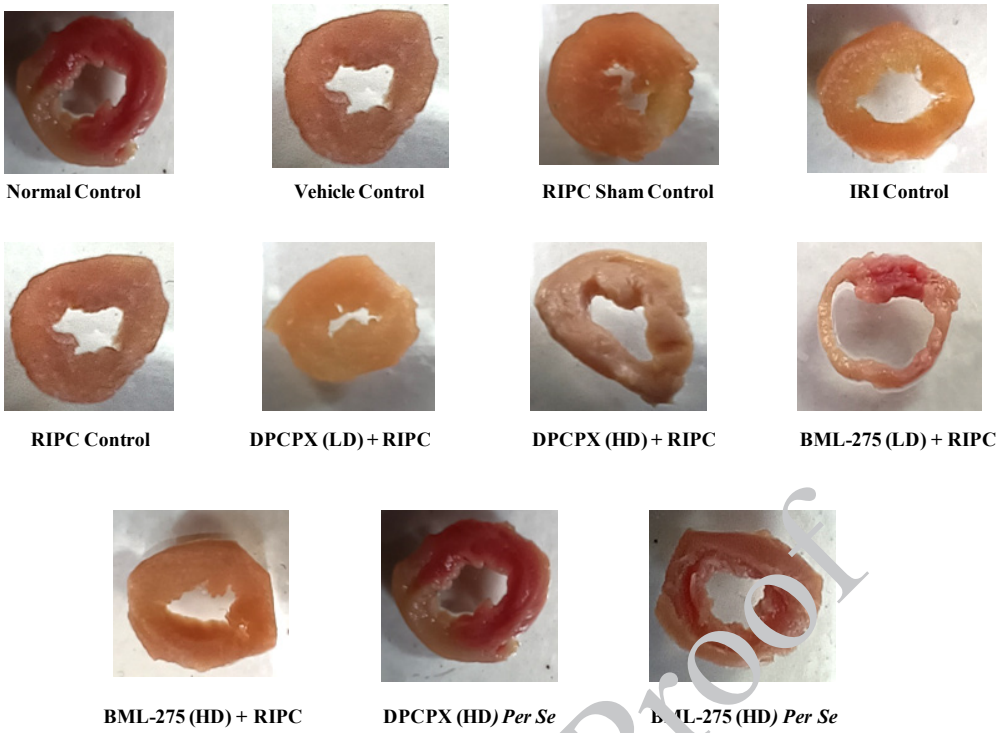

Figure 1. Microscopic depiction of the infarct size of rat heart sections utilizing the triphenyl tetrazolium chloride (TTC) staining method
The area stained yellow/white reflects the infarcted portion, whereas the area stained red colored reflects the viable portion in the rat hearts
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

Effects of different interventions on myocardial infarct size

A substantial increase in infarct size was observed in the IRI control group compared with the normal and vehicle control groups. Whereas RIPC substantially reduced infarct size compared with the IRI control group. Animals in the RIPC sham control group showed no discernible change in infarct size compared with the IRI control group. However, pre-treatment of DPCPX (AA_1R subtype selective antagonist; 0.1 and 0.2 mg/kg; *IP*) and BML-275 (AMPK-mediated autophagy inhibitor; 1.5 and 3 mg/kg; *IP*) significantly abrogated the myocardial protective effects of RIPC on infarct size as compared to the RIPC control group animals (Figures 1 and 2).

Effects of different interventions on specific myocardial injury markers

Effects on the activity of LDH-1 and CK-MB enzyme in coronary effluent samples

Prolonged ischemia for 30 min, followed by 120 min of reperfusion, markedly increased LDH-1 and CK-MB activities in coronary effluent samples collected at defined time intervals, compared with their basal values and with normal and vehicle control group animals. A substantial decline in LDH-1 and CK-MB activities was observed in the RIPC-treated group compared with the IRI control, whereas the RIPC sham group showed no significant change in these enzyme levels. However, pre-administration of DPCPX (AA_1R subtype selective antagonist; 0.1 and 0.2 mg/kg; *IP*) and BML-275 (1.5 and 3 mg/kg; *IP*) significantly attenuated the protective effects of RIPC on LDH-I and CK-MB activities as compared to the RIPC control group animals (Figures 3 and 4).

Effects on C-tPn-I protein content in the heart homogenate

A 30-minute ischemic period with subsequent reperfusion of 120 min caused a remarkable elevation in C-tPn-I protein levels in the myocardial injury animals as compared with the normal and vehicle control groups. In contrast, RIPC treatment markedly reduced C-tPn-I levels in the RIPC group relative to the IRI control group. On the contrary, RIPC sham group animals did not show any remarkable decline in the C-tPn-I protein content as compared to the animals of the IRI control group. However, pre-treatment of DPCPX (0.1 and 0.2 mg/kg; *IP*) and BML-275 (1.5 and 3 mg/kg; *IP*) significantly abolished the beneficial effects of RIPC on C-tPn-I protein content (Figure 5).

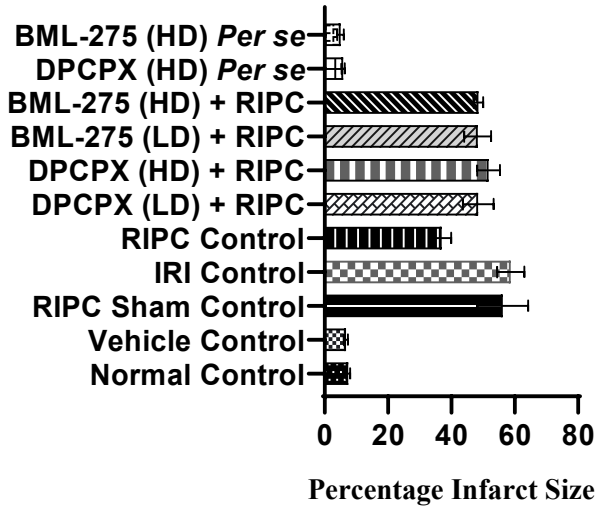

Figure 2. Effects of different interventions on rat heart infarct size
Final values are represented as mean±SD (n=6). a=P<0.05 vs Normal Control; b=P<0.05 vs Vehicle Control; c=P<0.05 vs IRI Control; d=P<0.05 vs RIPC Sham Control; e=P<0.05 vs RIPC Control
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

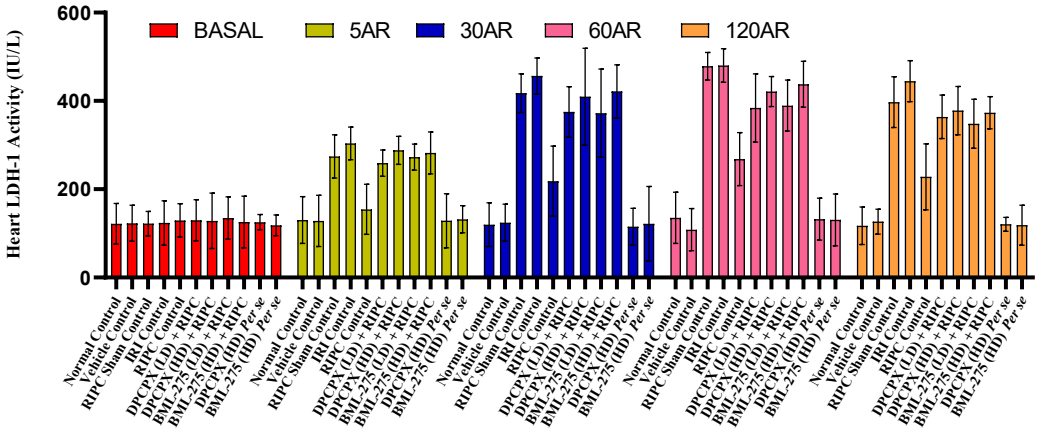

Figure 3. Effects of different interventions on activity of LDH-1 in rats
Final values are represented as mean±SD. *=*P*<0.05 vs Basal; a=*P*<0.05 vs Normal Control; b=*P*<0.05 vs Vehicle Control; c=*P*<0.05 vs IRI Control; d=*P*<0.05 vs RIPC Sham Control; e=*P*<0.05 vs RIPC Control
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

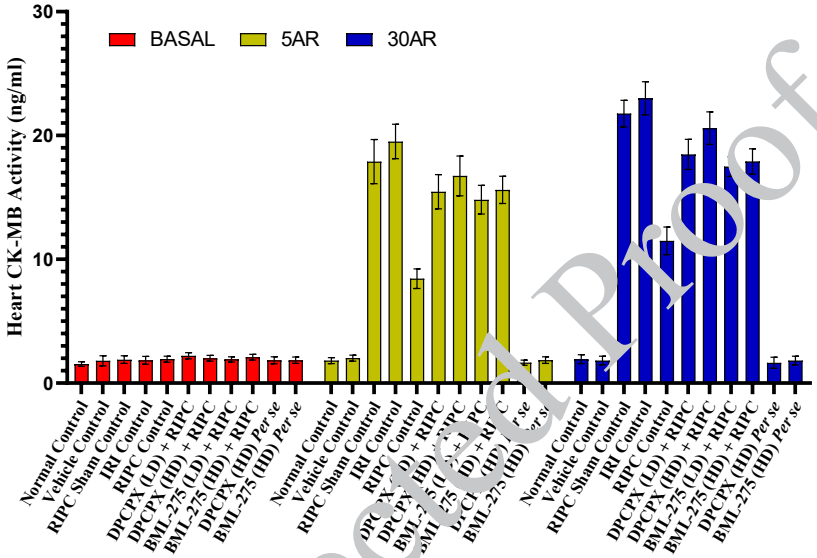

Figure 4. Effects of different interventions on the activity of C-MB
Final values are represented as mean±SD. *=*P*<0.05 vs Basal; a=*P*<0.05 vs Normal Control; b=*P*<0.05 vs Vehicle Control; c=*P*<0.05 vs IRI Control; d=*P*<0.05 vs RIPC Sham Control; e=*P*<0.05 vs RIPC Control
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

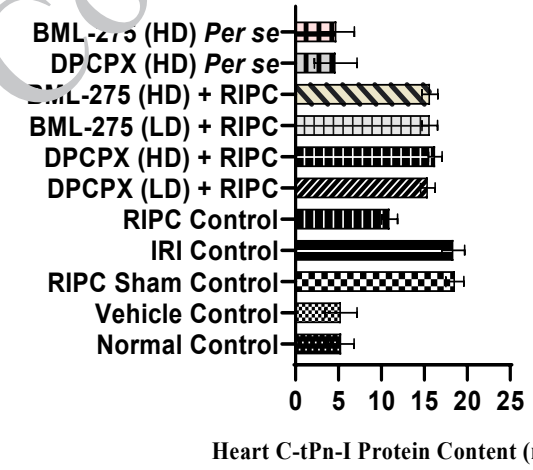

Figure 5. Effects of different interventions on protein content of C-tPn-I
Final values are represented as mean±SD. a=*P*<0.05 vs Normal Control; b=*P*<0.05 vs Vehicle Control; c=*P*<0.05 vs IRI Control; d=*P*<0.05 vs RIPC Sham Control; e=*P*<0.05 vs RIPC Control
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

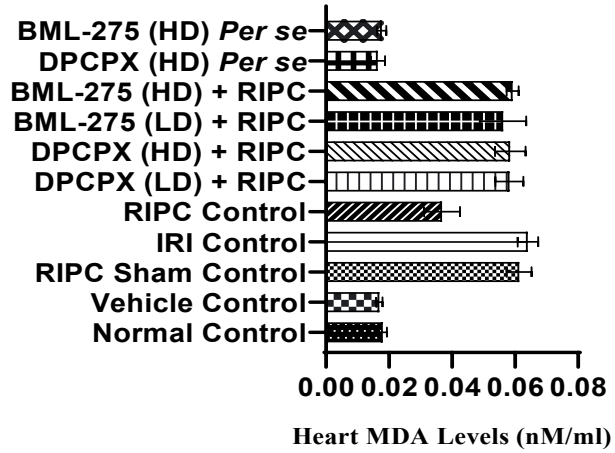

Figure 6. Effects of different interventions on levels of rat MDA
Final values are represented as mean±SD (n=6). a=*P*<0.05 vs Normal Control; b=*P*<0.05 vs Vehicle Control; c=*P*<0.05 vs IRI Control; d=*P*<0.05 vs RIPC Sham Control; e=*P*<0.05 vs RIPC Control
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

Effects of different interventions on markers of oxidative stress in the homogenate of the heart

Effects on GSH, catalase, and malondialdehyde levels in the homogenate of the heart

A pronounced elevation in MDA concentration (Figure 6), along with substantial reductions in GSH (Figure 7) and catalase (Figure 8) activities, was observed in the homogenates of the hearts of IRI control animals compared with the normal and vehicle control groups, reflecting increased oxidative stress. RIPC produced a substantial reversal of all aforementioned oxidative stress parameters compared with the IRI control group. On the contrary, animals of the RIPC sham group did not exhibit any significant improvement in the above-mentioned oxidative stress parameters as compared to the IRI control group animals. However, pretreatment with DPCPX (0.1 and 0.2 mg/kg; *IP*) and BML-275 (1.5 and 3 mg/kg; *IP*) significantly abrogated the protective effects of RIPC on all aforementioned oxidative stress markers (Figures 6-8).

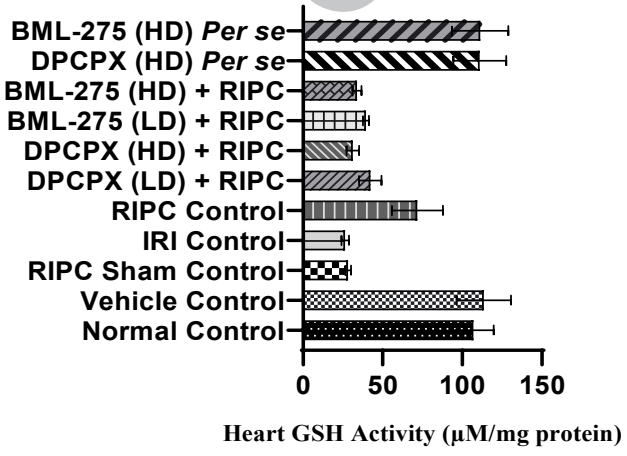

Figure 7. Effects of different interventions on the activity of GSH
Final values are represented as mean±SD (n=6). a=*P*<0.05 vs Normal Control; b=*P*<0.05 vs Vehicle Control; c=*P*<0.05 vs IRI Control; d=*P*<0.05 vs RIPC Sham Control; e=*P*<0.05 vs RIPC Control
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

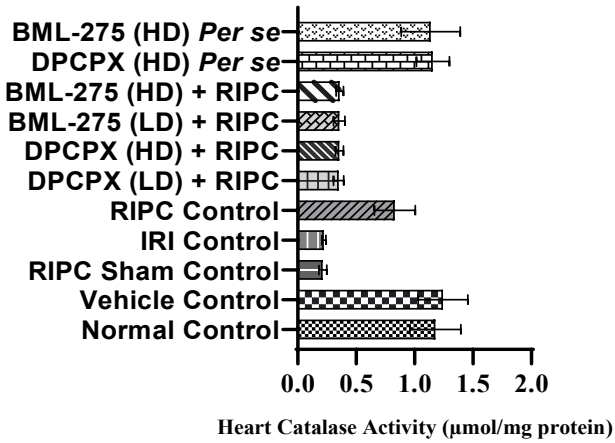

Figure 8. Effects of different interventions on the activity of catalase
Final values are represented as mean±SD (n=6). a=*P*<0.05 vs Normal Control; b=*P*<0.05 vs Vehicle Control; c=*P*<0.05 vs IRI Control; d=*P*<0.05 vs RIPC Sham Control; e=*P*<0.05 vs RIPC Control
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

Effects of different interventions on markers of inflammation and fibrosis

Effects on levels of TNF-α in the homogenate of the heart

Exposure to 30 min of prolonged ischemia followed by reperfusion of 120 min resulted in a pronounced rise in TNF-α concentration in the hearts of IRI control animals compared with the normal and vehicle controls. RIPC treatment effectively mitigated this elevation, while the RIPC sham group showed no considerable difference from the IRI control group. Pre-administration of DPCPX (0.1 and 0.2 mg/kg; *IP*) and BML-275 (1.5 and 3 mg/kg; *IP*) significantly abolished the protective effects of RIPC on TNF-α levels in comparison to the RIPC control group animals (Figure 9).

Effects on levels of TGF-β in the homogenate of the heart

A 30-minute period of sustained ischemia with subsequent reperfusion of 120 min resulted in a noticeable rise in cardiac TGF-β levels in the injury control group in comparison to normal and vehicle control groups. In

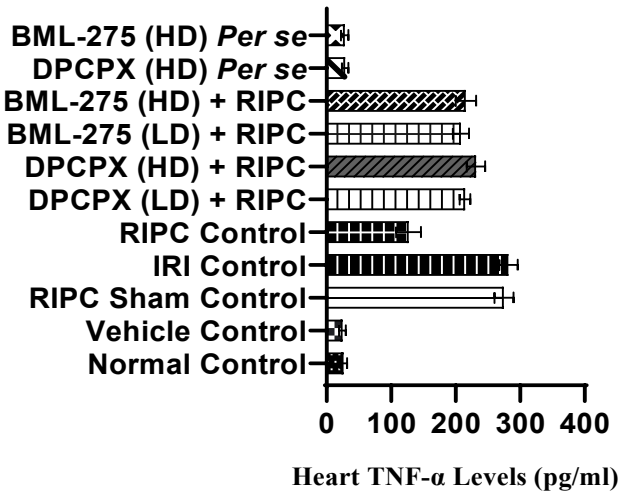

Figure 9. Effects of different interventions on levels of TNF-α in rats
Final values are represented as mean±SD (n=6). a=*P*<0.05 vs Normal Control; b=*P*<0.05 vs Vehicle Control; c=*P*<0.05 vs IRI Control; d=*P*<0.05 vs RIPC Sham Control; e=*P*<0.05 vs RIPC Control
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

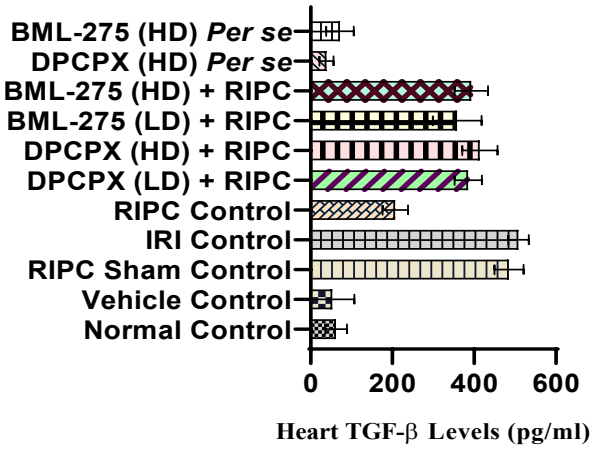

Figure 10. Effects of different interventions on levels of TGF-β in rats
Final values are represented as mean±SD (n=6). a=*P*<0.05 vs Normal Control; b=*P*<0.05 vs Vehicle Control; c=*P*<0.05 vs IRI Control; d=*P*<0.05 vs RIPC Sham Control; e=*P*<0.05 vs RIPC Control
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

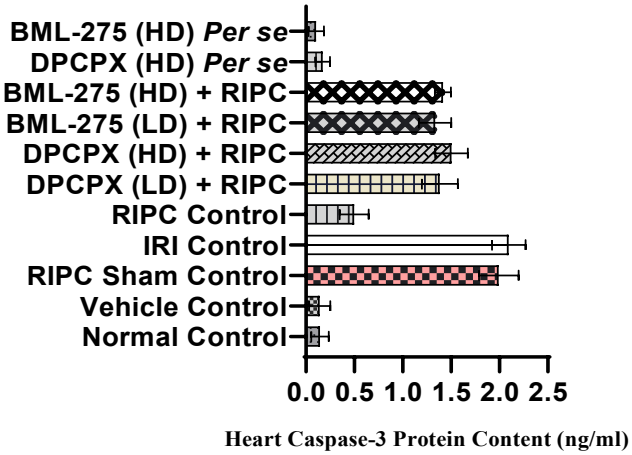

Figure 12. Effects of different interventions on the content of caspase-3 protein
Final values are represented as mean±SD (n=6). a=*P*<0.05 vs Normal Control; b=*P*<0.05 vs Vehicle Control; c=*P*<0.05 vs IRI Control; d=*P*<0.05 vs RIPC Sham Control; e=*P*<0.05 vs RIPC Control
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

contrast, RIPC treatment markedly reduced TGF-β levels relative to the IRI control group. However, the RIPC sham group did not exhibit any significant improvement in TGF-β levels compared with the IRI control group. However, pre-administration of DPCPX (0.1 and 0.2 mg/kg; IP) and BML-275 (1.5 and 3 mg/kg; IP) significantly abrogated the beneficial effects of RIPC on TGF-β levels (Figure 10).

Effects of different interventions on markers of apoptosis

Effects on Bax and Caspase-3 protein content in homogenate of heart

A 30-minute ischemic insult followed by 120 min of reperfusion significantly increased Bax and caspase-3 protein levels in the homogenates of the hearts of the myocardial injury control group compared with the normal and vehicle controls. RIPC treatment markedly reduced these apoptotic markers, whereas no significant changes were observed in the RIPC sham group relative to the IRI control group. However, pretreatment with DPCPX (0.1 and 0.2 mg/kg; IP) and BML-275 (1.5 and 3 mg/kg; IP) substantially reversed the protective effects of RIPC on Bax and caspase-3 protein levels compared with the RIPC control group (Figures 11 and 12).

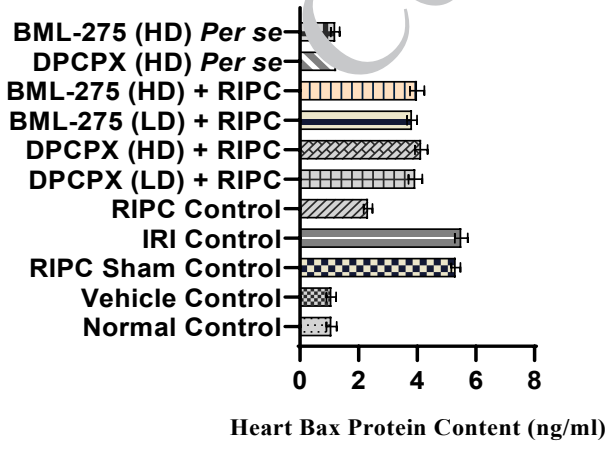

Figure 11. Effects of different interventions on the content of the Bax protein
Final values are represented as mean±SD (n=6). a=*P*<0.05 vs Normal Control; b=*P*<0.05 vs Vehicle Control; c=*P*<0.05 vs IRI Control; d=*P*<0.05 vs RIPC Sham Control; e=*P*<0.05 vs RIPC Control
RIPC: Remote ischemic preconditioning; IRI: Ischemia-reperfusion injury; DPCPX: Diphenyl cyclopentylxanthine

Discussion

The Langendorff digital power lab apparatus (AD Instruments, Australia) was used to induce MIRI, in which isolated rat hearts were subjected to 30 min of global ischemia followed by 120 min of retrograde reperfusion in KH buffer (27). The Langendorff perfusion model of the isolated heart is regarded as the benchmark *ex vivo* method for functional cardiac evaluation, enabling precise measurement of parameters such as HR, coronary perfusion, and LVDP (42).

In this study, MIRI was evaluated through multiple parameters such as myocardial infarct size, myocardial injury-specific biomarkers, and hemodynamic indices. Moreover, markers of oxidative stress, inflammation, fibrosis, and apoptosis were assessed to comprehensively determine cardiac injury and protection.

Sustained ischemia for 30 min followed by 120 min of reperfusion resulted in significant myocardial damage, as evidenced by an increased infarct size measured macroscopically using TTC staining. The myocardial ultrastructural damage observed in the IRI control group was consistent with previously documented studies (43). In addition to this, a substantial elevation in the activities of LDH-1 and CK-MB enzymes was detected in coronary effluent samples collected at designated time frames. Moreover, significant surge in C-tPn-I, TNF-α, TGF-β, Bax, caspase-3, and MDA levels, together with reduced GSH and catalase activities, was evident in cardiac homogenates. These results closely align with our earlier findings (5) and are supported by similar reports from other laboratories (44, 45).

Currently, four alternating phases of 5 min of ischemia followed by 5 min of reperfusion have been shown to produce remarkable cardioprotective effects against MIRI. RIPC markedly decreased the elevated infarct size, as well as the release of myocardial injury enzymes in the coronary

effluent. It also attenuated the increased levels of oxidative stress, inflammation, fibrosis, and apoptosis markers in the cardiac tissue homogenates. Furthermore, RIPC enhanced the reduced activities of antioxidant markers, indicating restoration of antioxidant defense. In addition, the cardioprotective effect of RIPC was evidenced by a substantial improvement in hemodynamic indices. As per previous findings, RIPC is effectively observed to reduce the extent of the infarct size, enhance left ventricular function, and mitigate edema and arrhythmias caused by reperfusion (46). Various preclinical (12, 47) and clinical (48) reports have documented that RIPC exerts cardioprotection against MIRI-induced damage.

In this study, the observed myocardial protective effect of RIPC was attenuated by pre-treatment with DPCPX, a selective AA_1R antagonist. It is well established that an imbalance in cellular energy homeostasis triggers the intracellular release of adenosine (13), which regulates several physiological processes, including coronary blood flow, heart rate, and myocardial contractility (4, 6, 49). Previous studies have also demonstrated that exogenous administration of adenosine confers protection against IRI by activating multiple metabolic pathways (14, 15). Moreover, activation of AA_1R has been reported to exert diverse protective effects, such as antioxidant, anti-inflammatory, and anti-apoptotic actions, along with preservation of mitochondrial function and enhancement of microvascular perfusion during IRI (50). Preclinical studies have further confirmed the beneficial role of AA_1R activation in experimental models of stroke and myocardial infarction (51, 52). Several investigations have also highlighted the crucial involvement of adenosine in mediating RIPC-induced protection across various organs, including the limb, kidney, mesentery, and carotid artery (8, 17). The alleviation of the cardioprotective effect of RIPC by AA_1R antagonist (DPCPX; 0.1 and 0.2 mg/kg; IP) in the present study implicates that AA_1R plays a crucial role in RIPC-mediated cardioprotection.

In this study, treatment with BML-275, an inhibitor of AMPK-dependent autophagy, nullified the cardioprotective effects of RIPC. Autophagy is a regulated cellular process that degrades and recycles damaged intracellular components (18). It occurs physiologically as well as pathologically, and common pathological conditions include nutritional deficiency, dys-homeostasis of energy metabolism, ischemia, hypoxia, and pathogen infection (19). It has been well documented that IRI attenuation is mediated by the induction of autophagy (20). Several cardioprotective interventions, including rapamycin (53), caloric restriction (54), and lipopolysaccharide administration (55), have been shown to stimulate autophagy. Furthermore, multiple signaling pathways, such as those involving protein kinase C, reactive oxygen species (ROS), nitric oxide (NO), and AMP-activated protein kinase (AMPK), are implicated in the regulation of autophagic activity. In addition, autophagy has been reported to be up-regulated during both ischemic and pharmacological preconditioning, where it contributes significantly to the observed cardioprotective effects (56, 57).

AMPK serves as a central regulator of cellular energy balance, responding to fluctuations in intracellular energy levels (22). Once activated, it phosphorylates various downstream effectors involved in metabolism and cell death. In the heart, AMPK-driven autophagy serves as a crucial defense mechanism that maintains cellular function under stress conditions such as IRI, oxidative stress, and nutrient scarcity (23, 24). Additionally, AMPK-mediated autophagy plays a pivotal role in the beneficial effects of RIPC against IRI by promoting cellular survival, reducing oxidative stress and inflammation, preserving mitochondrial function, and maintaining endothelial integrity. We found that the attenuation of cardioprotection conferred through RIPC by BML-275 (a selective inhibitor of AMPK-mediated autophagy; 1.5 and 3 mg/kg; IP) in MIRI. Current findings are directly witnessing that the cardioprotective effect of RIPC involves AMPK-activated autophagy.

Conclusion

The current study highlights that RIPC confers significant cardioprotection in MIRI through activation of AA_1R and downstream AMPK-mediated autophagy pathways. The attenuation of infarct size, restoration of hemodynamic index, and reduction of oxidative and apoptotic markers collectively highlight the mechanistic interplay between AA_1R activation and AMPK signaling as a pivotal adaptive response during ischemic stress. Moreover, RIPC, being a non-invasive and clinically applicable intervention, could serve as an adjunct strategy to reduce ischemia-reperfusion-induced cardiac injury in patients undergoing cardiac surgeries, angioplasty, or organ transplantation. Future studies integrating pharmacological AA_1R agonists with RIPC protocols may further optimize this dual pathway and advance its clinical applicability in personalized cardioprotection.

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None.

Availability of Data and Material (Data Transparency)

Animal data is available from the authors.

Ethics Approval (Include Appropriate Approvals or Waivers)

Experimental protocol was duly approved by the Institutional Animal Ethics Committee (Approval No: 107/Go/ReBi/S/99/CCSEA/2021).

Authors' Contributions

K K carried out the relevant research work and prepared the primary draft; N S conceived the idea, designed the research study protocol, and prepared the final draft of the article; HN Y provided valuable technical inputs in compiling the results and also corrected the primary draft of the manuscript.

Conflicts of Interest

Authors declare that there is no conflict of interest.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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