Pretreatment with Nicotinamide Mononucleotide Increases the Effect of Ischemic-Postconditioning on Cardioprotection and Mitochondrial Function Following ex vivo Myocardial Reperfusion Injury in Aged Rats

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**Running title:** Combination of nicotinamide-mononucleotide and postconditioning improves protection of aged heart

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The present study aims to evaluate the combined effect of ischemic-postconditioning (IPostC) and nicotinamide mononucleotide (NMN) on cardioprotection and mitochondrial function in aged rats subjected to myocardial ischemia-reperfusion (IR) injury. Sixty aged Wistar rats were randomly divided into 5 groups (n=12), including sham, control, NMN, IPostC, and NMN+IPostC. Regional ischemia was induced by 30-min occlusion of the left anterior descending coronary artery (LAD) followed by 60-min reperfusion. IPostC was applied at the onset of reperfusion, by 6 cycles of 10-s reperfusion/ischemia. NMN (100 mg/kg) was intraperitoneally injected every other day for 28 days before IR. Myocardial hemodynamics and infarct size (IS) were measured, and the left ventricles samples were harvested to assess cardiac mitochondrial function. The results showed that all treatments reduced lactate dehydrogenase release compared to those of the control group. IPostC alone failed to reduce IS and myocardial function. However, NMN and combined therapy could significantly improve myocardial function and decrease the IS compared to the control animals. Moreover, the effects of combined therapy on the decrease of IS, mitochondrial reactive oxygen species (ROS), and improvement of mitochondrial membrane potential (MMP) were greater than those of alone treatments. These results demonstrated that cardioprotection by combined therapy with NMN+IPostC was superior to individual treatments, and pretreatment of aged rats with NMN was able to correct the failure of IPostC in protecting the hearts of aged rats against IR injury.

**Keywords:** Aging, Ischemic postconditioning, Mitochondrial function, Nicotinamide
1|Introduction

Ischemia is the main cause of myocardial tissue damage because of the rapid decline in the supply of oxygen and nutrients to the myocardium. Early reperfusion has a crucial effect on reducing heart muscle damage and protects the pumping function in the ischemic heart 1,2. Nevertheless, reperfusion can exacerbate the myocardium injury, leading to induction of arrhythmias, no-reflow phenomenon, and myocardial dysfunction and stunning, along with microvascular damage 1-4.

Aging is a critical risk factor of cardiovascular diseases (CVD), which represents 30% of all deaths worldwide 5. Moreover, aging affects the modification of myocardial and vascular function, even in the absence of any pathologic conditions 5,6. Experimental and clinical findings have shown that the aged heart is more vulnerable to myocardial ischemia and reperfusion (IR) injury 6,7. Even after well-timed and successful reperfusion, the mortality rate and myocardial damage are higher in elderly patients 5. Myocardial injury in isolated, buffer-perfused hearts of aged rats (24-month) was higher than in control adult rats (6-month) following IR injury 6,7. The increased cardiac damage in the aged heart of other strains 8 and species of rats 9 has been also reported. The senescent heart is more vulnerable to metabolic insults as several animal species have shown greater susceptibility to IR injury related to hemodynamic factors, collateral flow, and damaging cellular elements in the blood of the reperfused heart 10-14. More importantly, the senescent heart is more resistant to protection against IR injury 14.

A controlled reperfusion technique called ischemic postconditioning (IPostC) is the cycles of brief periods of reperfusion and ischemia applied after a long period of ischemic insult that can improve the ischemic outcome significantly 15,16. It appears that IPostC is very effective in reducing IR injury in young animals by preventing mitochondrial damage via closing mitochondrial permeability transition pore (mPTP) 16. Using IPostC in reperfusion of long-time post-ischemia may be considered as a novel approach for cardioprotection 17,18. IPostC initiates endogenous protective responses in the heart with the expression of protective and stress-responsive proteins that are responsible for cardioprotection 19,20. Also, IPostC has been shown to limit the infract size (IS) and induce cardioprotection by preventing reperfusion-induced lethal pathways 21-23. On the other hand, the cardioprotective roles of IPostC against IR injury have been shown in young hearts but not in the aged hearts 18,19. One reason that IPostC fails to induce
cardioprotection in aging is that main surviving end effectors such as mitochondria in aged cardiomyocytes may be dysfunctional and therefore application of IPostC alone may not be able enough to activate survival mediators.

Nicotinamide mononucleotide (NMN) is an intermediate nucleotide for the biosynthesis of nicotinamide adenine dinucleotide (NAD+) as an essential mitochondrial coenzyme in all living cells \(^4\) that has shown beneficial physiological effects in cardioprotection. NMN has an anti-aging effect by enhancing cellular biochemical functions in Alzheimer's disease and obesity-related complications \(^24\). In healthy cells, the loss of NAD\(^+\) leads to inhibition of cellular respiration, and consequently, a loss of mitochondrial function and ATP production, and finally cellular death \(^25,26\). Brain ischemia leads to a critical NAD\(^+\) depletion and consequently, brain cell death, and replenishment of NAD\(^+\) provided remarkable neuroprotection against ischemic cell death \(^27\). In an animal study, a period of NMN administration intraperitoneally (500 mg/kg) 30 min before starting ischemia caused a reduction of infarct size up to 44%, while its administration every 6 hours during the reperfusion phase for 24 hours reduced the infarct size up to 29\% \(^28\).

Evidence shows that necessary cofactors for mitochondrial enzymes function, such as NAD\(^+\), are decreased by advancing age and during ischemia, leading to mitochondrial dysfunction. Moreover, previous studies have shown that the application of the IPostC alone cannot be effective in treating ischemic heart disease in the elderly \(^19,29\). It seems that boosting the mitochondrial activity before ischemia in aged hearts may increase the potency of protective modalities during reperfusion. As a consequence, the purpose of our study is to examine the effect of combining IPostC and NMN on cardioprotection in aged animals by focusing on cardiac hemodynamic, infarct size, and lactate dehydrogenase (LDH) measurements accompanied by mitochondrial reactive oxygen species (ROS) generation and mitochondrial membrane potential (MMP) changes in IR injury. We hypothesized that improving mitochondrial function via restoration of NAD\(^+\) level by using NMN before ischemia can increase the cardioprotection efficiency of IPostC in the aged heart.

2|Results

2.1|Hemodynamic Parameters
2.1.1|Coronary flow

In this study, the effects of NMN preconditioning and IPostC, alone or in combination, on cardioprotection of aged hearts following IR injury were evaluated. In the ischemic phase, the coronary flows (CFs), measured by time-collection of coronary effluents and adjusted for heart weights, were lower in all of the groups with IR injury in comparison to their baseline values and the sham group (Figure 1). Reperfusion of aged ischemic hearts increased the CFs in all groups, but there was no significant difference among all groups.

2.1.2|Heart Rate

Figure 2 displayed that the heart rates (HRs) at the ischemic phase were significantly lower in all aged IR hearts versus sham group (P < 0.001 at 15 and 30 minutes of ischemia). After reperfusion of the ischemic hearts in all groups, the HR was increased. However, there were no significant differences in HRs before IR and during the reperfusion phase between groups.

2.1.3|Left Ventricular Developed Pressure

Analysis of the data showed that induction of regional ischemia via LAD ligation for 30 min decreased developed pressures of the left ventricles (LVDP), measured by ventricular balloon connected to a pressure transducer, in all groups exposed to IR injury (Figure 3). The LVDP in the NMN and combination group (NMN+IPostC) groups was significantly greater than the control group throughout the reperfusion period (P < 0.001). In addition, the post-hoc test showed that the increasing effect of combination therapy on LVDP was significantly greater than that of the NMN alone group at 90th min of reperfusion (P < 0.05) and the IPostC group had significantly lower LVDP than NMN and NMN+IPostC group (P < 0.001).

2.1.4|Left Ventricular End-Diastolic Pressure

The end-diastolic pressure of the left ventricles (LVEDPs) was noticeably elevated in the IR aged hearts during ischemic and reperfusion phases in comparison to the baselines (P < 0.001, Figure 4). Moreover, the LVEDP was significantly higher in the treatment groups compared to the sham group. Although prior administration of NMN for 28 days significantly reduced the LVEDP at different time points of reperfusion as compared with the control group, application of
IPostC was not able, as much as NMN, to prevent the elevation of LVEDP during reperfusion. In addition, concomitant administration of both NMN and IPostC markedly diminished LVEDP (P < 0.001) as compared with control hearts. Compared with the control group, the effect of combination therapy on reduction of LVEDPs decreased was greater than that of NMN alone treatment at 45th and 75th mins of reperfusion (Figure 4).

2.2|Infarct Size

Myocardial infarct sizes (IS) and areas at risk were determined via the TTC staining method and planimetry. There were no significant differences in the volumes of the area at risk of the myocardium between groups (Figure 5A). Both NMN-pretreatment alone and a combination of IPostC and NMN significantly reduced the sizes of infarction (IS) of the aged animals to 25.7% + 2.14% and 21.2% + 1.54%, respectively, as compared to those of the control group (P < 0.001). Although the effect of combination therapy was somewhat greater than that of NMN, the difference between them was not statistically significant. In addition, IPostC was tended to reduce IS of aged hearts in comparison to those of the control group but this effect was not statistically significant. Compared with the IPostC group, IS was significantly lesser in the NMN (P < 0.01) and NMN+IPostC groups (P < 0.001) (Figure 5B).

2.3|LDH release

The release of LDH, measured spectrophotometrically, was significantly increased in the control hearts versus the sham group (P < 0.001). Both application of IPostC (P < 0.01) at the beginning of reperfusion and pretreatment of NMN (P < 0.001) in the aged rats significantly blocked the LDH release in comparison with the control group. Moreover, combination therapy markedly decreased myocardial LDH release as compared to the control group (P < 0.001, Figure 6). The effect of combination therapy in reducing LDH levels was significantly greater than those of single therapy (P < 0.01, and P < 0.05).

2.4|Mitochondrial Profile

2.4.1|Cardiac Mitochondrial ROS Assessment
Intergroup analysis showed that IR injury significantly increased ROS level, evaluated freshly at the end of reperfusion through DCFDA staining, as compared to the sham group (P<0.001). Administration of NMN, IPostC, or their combination considerably decreased mitochondrial ROS levels (P<0.01, P<0.001, and P<0.001, respectively) versus the control group (Figure 7A). In addition, the beneficial effects of IPostC+NMN on mitochondrial ROS were significantly greater than those of NMN alone (P<0.01) and IPostC alone (P<0.01).

### 2.4.2 Cardiac mitochondrial membrane potential changes

Our results showed that IR injury in aged hearts significantly decreased the mitochondrial membrane potential [MPP] (P<0.001), assessed via JC-1 staining, in comparison to the sham group (Figure 7B). In other words, induction of myocardial IR injury in aged rats significantly depolarized the mitochondrial membrane. Administration of IPostC (P<0.05) or NMN (P<0.001) separately or in combination (P<0.001) could increase the MMP compared to control hearts. Moreover, the effect of combination therapy on MMP changes was significantly greater than that of IPostC alone treatments (P<0.001) (Figure 7).

### 3 Discussion

The results of this study demonstrated that IPostC was unable to induce cardioprotection in aged hearts. However, pretreatment of aged rats with NMN had significant preventive effects on IR-induced cardiac dysfunction and infarct size as well as mitochondrial function. Moreover, the cardioprotective effect of IPostC on aged hearts was considerably recovered in aged rats pretreated with NMN. As a result, combination therapy with IPostC and NMN had greater protective impacts against myocardial IR injury, as shown by further improvement of cardiac function, much smaller infarct size and LDH release, lower mitochondrial ROS production, and less mitochondrial membrane depolarization.

Mitochondrial dysfunction is one of the main consequences of myocardial IR injury that induces a variety of intracellular adverse events, leading to cardiac contractile dysfunction and cell death. During the ischemic phase, deprivation of oxygen and glucose causes mitochondrial damage. The reperfusion also drives overproduction of mitochondrial ROS and depletion of ATP production, exacerbating myocardial injury. The calcium overload and generation of free radicals in the mitochondria facilitate the opening of mitochondrial permeability transition pore...
which consequently leads to loss of membrane potential, excessive production of ROS, transferring cytochrome C and apoptotic factors into the cytosol, and activation of cell death pathways. Accordingly, our results showed that induction of IR damage in aged hearts led to cardiac dysfunction, considerable development of infarct size and LDH release as well as mitochondrial ROS overproduction and membrane depolarization, whereas, administration of NMN with or without IPostC restored these indices. It has been documented that besides its antioxidative capacities, NMN acts as a protective mitochondrial cofactor, increasing its biogenesis and function. Its cardioprotective impacts and some of the underlying mechanisms have been shown to some extent in young animals previously. For example, Yamamoto et al. have reported that exogenous administration of NMN to young rats exerted cardioprotective effects via decreasing FOXO1 acetylation, and increasing SIRT1 activity and autophagy flux. Increased activity of FOXO1 and SIRT1 ultimately leads to improved mitochondrial function. Also, alone treatment with NMN has effectively reduced myocardial IR-induced LDH release in aged rats. We demonstrated again the cardioprotective and LDH-lowering effects of NMN in aged rats, which these effects were associated with its mitochondrial boosting potentials.

More importantly, IPostC alone could not significantly improve cardiac function and reduce infarct size, indicating the ineffectiveness of this therapeutic strategy in aging conditions despite its positive effects on mitochondrial parameters. IPostC is an endogenous protection mechanism that exerts greater cardioprotection in young animals. According to previous reports, its potency may be dramatically attenuated in aged hearts due to the aging-induced pathophysiological conditions in cardiomyocytes such as mitochondrial dysfunction, DNA mutations, dysregulation in the gene/protein expression, and increased oxidative stress and inflammatory responses. Our findings confirm the previous evidence that IPostC failed to provide cardioprotection in aged rats as it did in young animals. Though IPostC alone had no significant effect on infarct size, it reduced LDH release in aged rats. This likely suggests that LDH release occurred independently of the myocardial infarction following IR injury in the aged rats.

It has also been suggested that aging-associated mitochondrial dysfunction may play a central role in decreased response to interventions such as IPostC. Here, IPostC acts via the mitochondria as the main end-effector. Therefore, it seems that improving mitochondrial function may potentiate the cardiac response to IPostC intervention in aging. According to this hypothesis in the present study, NMN was administered to aged rats before inducing I/R injury and applying IPostC. Administration of NMN for 28 days was able to restore mitochondrial function. It is worth noting that NMN administration at the beginning of reperfusion has
almost the same effects as its pre-ischemic administration in young rats. Since aging is associated with a decline in the function of vital organelles, namely mitochondria, and IR injury leads to further mitochondrial damage, we firstly administered NMN before ischemia to aged rats to partially compensate for the mitochondrial dysfunction and other intracellular conditions induced by aging, and then evaluate IPostC effectiveness in aged hearts. As a result, the strongest cardioprotective effect was obtained in a group of aged rats that received concomitant administration of NMN plus IPostC. The effect of this combination therapy was significantly greater than that of IPostC in all parameters and that of NMN on cardiac hemodynamics such as LVDP at the end of reperfusion, LDH release, or mitochondrial ROS levels. Thus, despite the ineffectiveness of IPostC alone in protecting the heart against IR damage, its effectiveness increased significantly just after prior administration of NMN. These findings support our hypothesis that age-related dysfunction in cardiac cells and their mitochondria impede the effect of interventions that have stronger effects in young rats. It is also inferred that a combination of interventions may have adequate efficacy in protecting aged hearts against IR injury.

Our results confirmed that NMN alone is effective in protecting the aged heart, but when used concomittantly with IPostC, their effects are multiplied, and in a way, NMN pretreatment restored the failure of IPostC in protecting the aged heart. Thus, pretreatment with NMN may modulate negative cellular alterations in aged hearts before inducing IR through enhancement of cellular antioxidant machinery and recycling of NAD$^+$ which eventually improve mitochondrial function. Accordingly, the cardioprotective effect of combination therapy was accompanied by improvement in mitochondrial function. Reduction of mitochondrial ROS in IR hearts following combined intervention indicates that the mitochondrial redox has returned to a more favorable status, and this leads to better mitochondrial function and promotes its biogenesis. Modification of mitochondrial membrane potential and prevention of membrane depolarization can be evidence of better mitochondrial function. However, the reduction of mitochondrial ROS level and its membrane depolarization following IPostC alone beside the lack of its cardioprotective impacts highlights that there may be additional survival mediators other than mitochondria, mediating the protective effects of combination therapy in the aging heart. In addition to mitochondrial-dependent effects, NMN may possess several non-mitochondrial-dependent properties, including anti-apoptotic, anti-inflammatory, and pro-autophagic effects, all of which are protective. Above and beyond, the cardioprotective effects of this combination may be achieved through the activation/inhibition of mediators and signaling pathways such as...
PI3K/Akt/GSK-3β, autophagy/mitophagy, mitochondrial potassium channels and biogenesis pathways, and mPTP which further studies are warranted to elucidate their contribution.

Finally, it should be noted that combination therapies in young rats do not necessarily have greater effects than single therapies. The most likely hypothesis in this regard is that each of the effective interventions in young rats exerts almost their maximal effects in activating protective mechanisms, so the additive effects are not significant in their combination. However, in older animals, the therapeutic efficacy of individual therapies is insufficient for the reasons described, so their combination can overcome the interfering effects of aging by activating similar or parallel pathways and produce stronger protection than individual conditionings. Besides mitochondrial dysfunction, more mechanisms need to be studied to elucidate the reasons for the reduced response to individual conditionings in aging.

Conclusion

Our findings indicated that pretreatment of aged rats with NMN before IR insult plus the application of IPostC at the onset of reperfusion induced more cardioprotective effect and significantly reduced infarct size, improved cardiac hemodynamic, attenuated mitochondrial ROS, and restored MMP. IPostC alone failed to improve cardiac function and limit infarct size in aged rats. NMN pretreatment was able to correct the failure of IPostC in protecting the hearts of aged rats against IR injury (Figure 8). Mechanisms other than preservation of mitochondrial function may contribute to the combination therapy-induced cardioprotection in the aged rats which need to be clarified in the future.

4|Material and methods

4.1|Animals and drug administration

A total of sixty (22-24 months old) male Wistar rats weighing between 400-450 g were purchased from the Animal Center of Tabriz University of Medical Sciences and used in this study. Animals were housed in standard polypropylene cages, there per cage, and all animals were kept under a 12:12 light/dark schedule, at a temperature of 25 ± 2 °C and a humidity of 55%. They had unlimited access to standard pellet food and tap water. All of the procedures were conducted under the ethical guidelines of the National Institutes of Health (NIH) for the use and care of laboratory animals and approved by the Ethical Committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1396.395).
After 2 weeks of acclimatization, all experimental rats were randomly divided into 5 groups (n=12/each): 1) Sham: the hearts were exposed to 90 min perfusion without ischemia, 2) control: the hearts were exposed to 30 min ischemia + 60 min reperfusion, 3) NMN: the hearts of NMN receiving rats were exposed to 30 min ischemia + 60 min reperfusion, 4) IPostC: the hearts were exposed to 30 min ischemia + 6 cycles of 10s R/I at the beginning of 60 min reperfusion, and 5) NMN+ IPostC: the hearts of NMN receiving rats were exposed to 30 min ischemia + 6 cycles of 10s R/I at the beginning of 60 min reperfusion. NMN (Sigma, USA) was administered intraperitoneally at the dose of 100 mg/kg/day (dissolved in sterile saline) every other day for 28 days, before induction of myocardial ischemia. Rats in NMN untreated groups also received similar amounts of sterile saline intraperitoneally at similar time points. Six hearts from each group were used for freshly mitochondrial measurements and the other six hearts for IS measurement.

4.2 | Surgery for isolated perfused hearts preparation

The animals were fasted eight hours before surgery. Following the heparinization of rats (500 IU), they were anesthetized by an injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). After thoracotomy, the heart was quickly isolated and then transferred to the Langendorff isolated hearts apparatus with a constant pressure mode at 80 mmHg. Then, the hearts were perfused in a retrograde manner through the aortic cannula with the Krebs-Henseleit solution at pH 7.4 and a constant temperature of 37°C. The perfusion solution contained (in mmol/l: NaCl, 118; KCL, 4.7; KH2PO4, 1.2; MgSO4, 1.2; CaCl2, 2.5; NaHCO3, 25; and glucose, 11.1) and bubbled with carbogen containing 95 % O2 and 5 % CO2.

4.3 | Ischemic reperfusion experimental protocol

At first, the isolated hearts were perfused with Krebs-Henseleit solution for 15 minutes as a stabilization period. Then, regional ischemia was induced for 30-min by ligation of the left anterior descending (LAD) coronary artery, and followed by reperfusion period for 60-min by reopening of LAD. The same procedure was performed on the sham-operated group with the exception that the ligature was left untied. To determine the adequate perfusion and coronary ligation efficiency, the coronary flow (CF) was measured in this study. During LAD closure, at least a 25% reduction in CF was considered satisfactory. Furthermore, in IPostC receiving
groups, 6 cycles of 10-seconds reperfusion/ischemia were induced in hearts at the beginning of reperfusion\textsuperscript{18,42}. After 60-min of reperfusion, the heart tissues of six rats in each group were harvested for mitochondrial analyses. Moreover, the IS assessment was performed 60-min post-reperfusion on another six rats of each group.

4.4|Heart hemodynamics measurement

During the stabilization of the heart on the Langendorff apparatus, a latex balloon (Harvard Apparatus Ltd, Eden Bridge, United Kingdom) was attached to a stiff polyethylene tube ending and inserted into the left ventricle (LV) via the mitral valve. After connecting the balloon and tube to a pressure transducer, it was filled with normal saline to produce a left ventricular end-diastolic pressure (LVEDPs) that adjusted between 5-10 mmHg. The Power Lab data acquisition system (AD Instruments, Newcastle, NSW, Australia) was used to measure heart rates (HRs), LV-developed pressures (LVDPs), and LVEDP. All hemodynamic variables were recorded continuously in all groups during the experimental procedure. To measure the CFs, the coronary effluent was collected throughout the experiment and presented as L/min\textsuperscript{42,43}.

4.5|Infarct size evaluation

At the end of the 60-min reperfusion period, the LAD coronary artery was occluded again and followed by an infusion of 2 ml Evans blue dye (0.25\%) into coronary circulation. Then, a 2-mm-thick cross-section was prepared from the frozen hearts and immersed in 1\% 2,3,5-triphenyl tetrazolium-chloride phosphate-buffered solution (PBS), pH 7.4 at 37 °C for 15-min. Then, to evaluate the staining contrast, the slices were placed in formalin solution (10\%, for 24 hours). To measure the infarct volume, Image-J software was employed by a blinded investigator to the animal groups. Percentages of the LV and area at risk (AAR) volumes were measured to report the AAR and ISs, respectively.

4.6|Measurement of Lactate Dehydrogenase release

To evaluate the myocardial injury, LDH levels were measured in coronary effluent during the reperfusion period. Plasma levels of LDH were determined by a standard enzyme-linked immunosorbent assay (ELISA) kit (Pars-Azmoon Co, Tehran, Iran), and activities of the enzyme
were adjusted based on the weights of the hearts and presented as U/gram tissue. All experimental procedures were performed by following the kit's instructions.

4.7 | Isolation of cardiac mitochondria

For isolation of mitochondria, at the end of reperfusion, the LV in the aged myocardium was rapidly harvested. About 100 mg myocardial AAR was minced and homogenized in solution buffer containing: 200 mmol/L mannitol, 1 mmol/L EDTA in 50 mmol/L Tris/HCl, 70 mmol/L sucrose, 10 mmol/L HEPES, pH 7.4, and 4°C. After centrifuging the homogenate at 1300 g for 3-min, the supernatant was re-centrifuged at 10000 g for 10-min. To suspend the mitochondrial pellet, a 100 mL storage buffer was used. The content of storage buffer was 250 mM sucrose, 1 mM ATP, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 5 mM sodium succinate, 0.08 mM adenosine diphosphate, 1 mM dithiothreitol, and 2 mM K2HPO4. Protein content in mitochondria assayed using Nanodrop (Thermo Fisher Scientific, Wilmington, USA) 44.

4.8 | Cardiac mitochondrial ROS generation

For evaluation of the mitochondrial ROS production, the mitochondrial pellets were incubated with 40 mL dichlorodihydrofluorescein diacetate (DCFDA) dye for 30-min at room temperature. DCFDA, a fluorogenic dye, measures ROS activity within the cell organelles and after diffusion into organelle is oxidized by ROS into 20,70-dichlorofluorescein (a highly fluorescent compound). The fluorescence intensities (FIs) were detected at wavelengths of 480 nm and 530 nm by a fluorometric method. An elevation in the FL indicated increasing the mitochondrial ROS level and was expressed as FI/mg of protein 45.

4.9 | Cardiac mitochondrial membrane potential changes

To measure cardiac mitochondrial membrane potential (MMP) changes, the mitochondria staining JC-1 dye (Sigma-Aldrich) was used according to the manufacturer’s protocol. Isolated mitochondria were diluted in JC-1 stain (2.5 mg/mL) and incubated in the dark at room temperature for 30-min. JC-1 aggregates (red fluorescent, in normal condition), were detected in excited and an emission wavelength of 525 and 590, respectively. JC-1 monomers (green
fluorescent) in unhealthy cells were excited at 458 nm and their emission was detected at 530 nm wavelength. A decrease in the red/green FI ratio was representative of cardiac mitochondrial membrane depolarization. A spectrofluorometer was used to record FL 45.

4.10|Statistical Analysis

All descriptive data were expressed as means ± SEM. Repeated measures analysis of variance (ANOVA) was used to analyze the hemodynamic data, and one-way ANOVA was used for other parameters and followed by the post hoc Tukey test to determine the statistical significance between groups. All analysis procedures were performed using Graph Pad Prism (version 6.01), and p<0.05 was considered statistically significant.

5|Acknowledgments

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6| Declaration of Conflicting Interests

The authors have declared that they have no conflicts of interest.

7| Data Availability Statement

Data are available upon request from authors.

8| References


Figure legends

Figure 1. Coronary flow in aged hearts. The data were from 6 independent experiments (n=6/each group) and were expressed as mean + standard error of the mean (S.E.M). Symbol *** represents significant difference at P < 0.001 in all experimental groups versus Sham group. NMN: nicotinamide mononucleotide, IPostC: ischemic postconditioning.

Figure 2. Heart rates in aged hearts. The data were from 6 independent experiments (n=6/each group) and were expressed as mean + standard error of the mean (S.E.M). Symbol *** represents significant difference at P < 0.001 in all experimental groups versus Sham group. NMN: nicotinamide mononucleotide, IPostC: ischemic postconditioning.

Figure 3. The left ventricular developed pressure (LVDP). The data were from 6 independent experiments (n=6/each group) and were expressed as mean + standard error of the mean (S.E.M). ***P < 0.001 (for all groups at ischemic phase) versus Sham; ###P < 0.001 versus Control group; $P < 0.05, and $$$P < 0.001 versus IPostC group; $P < 0.05 versus NMN group. Symbols and curves of the same color are related to each other in reperfusion phase. NMN: nicotinamide mononucleotide, IPostC: ischemic postconditioning.

Figure 4. The left ventricular end-diastolic pressure (LVEDP). The data were from 6 independent experiments (n=6/each group) and were expressed as mean + standard error of the mean (S.E.M). ***P < 0.001 (for all groups at ischemic phase) versus Sham; ###P < 0.01, and ####P < 0.001 versus Control group; $P < 0.01, and $$$P < 0.001 versus IPostC group. Symbols and curves of the same color are related to each other in reperfusion phase. NMN: nicotinamide mononucleotide, IPostC: ischemic postconditioning.

Figure 5. Area at risk (AAR; A) and infarct sizes (B) percentages in the aged hearts. The data were from 6 independent experiments (n=6/each group) and were expressed as mean + standard error of the mean (S.E.M). ###P < 0.001 versus Control group; $$P < 0.01 and $$$P < 0.001 versus IPostC group. NMN: nicotinamide mononucleotide, IPostC: ischemic postconditioning.

Figure 6. The levels of lactate dehydrogenase (LDH) release into the coronary effluent in the aged hearts. The data were from 6 independent experiments (n=6/each group) and were expressed as mean + standard error of the mean (S.E.M). ***P < 0.001 versus Sham; ###P < 0.01
and ###P < 0.001 versus Control; $$P < 0.01$$ versus IPostC group; @P < 0.05 versus NMN group. NMN: nicotinamide mononucleotide; IPostC: ischemic postconditioning.

**Figure 7.** (A) Mitochondrial reactive oxygen species (ROS) production level and (B) Cardiac mitochondrial membrane potential (MMP) change in the aged hearts. The data were from 6 independent experiments (n=6/each group) and were expressed as mean ± standard error of the mean (S.E.M). ***P < 0.001 versus Sham; #P < 0.05, ##P < 0.01, and ###P < 0.001 versus Control group; $$P < 0.01$$ and $$$P < 0.001$$ versus IPostC group; @P < 0.05 versus NMN group. NMN: nicotinamide mononucleotide, IPostC: ischemic postconditioning.
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The image shows a bar graph comparing LDH release (U/g of tissue) across different groups: Sham, Control, NMN, IPostc, NMN+IPostc. The graph indicates significant differences between the groups, with NMN showing a notable decrease compared to the control group. The specific statistical significances are marked with symbols: three asterisks for the control group compared to Sham, two asterisks for NMN compared to control, and two symbols each for IPostc and NMN+IPostc compared to control. The graph suggests that NMN and the combination NMN+IPostc reduce LDH release more than the control group.