Hypercholesterolemia attenuates cardioprotection of ischemic preconditioning and postconditioning with α7 nicotinic acetylcholine receptor agonist by enhancing inflammation and inhibiting the PI3K/Akt/eNOS pathway

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Abstract. The present study aimed to evaluate the effects of hypercholesterolemia on cardioprotection of ischemic preconditioning and α7 nicotinic acetylcholine receptor (α7nAChR) agonist postconditioning and explore the potential mechanisms that hypercholesterolemia affected their cardioprotection. Hypercholesterolemic and normal rats were divided into the four groups that received the following treatments: i) Hypercholesterolemic control and normal control groups; ii) hypercholesterolemic ischemia/reperfusion (HI) and normal ischemia/reperfusion (NI) groups; iii) hypercholesterolemic ischemic preconditioning (HIPC) and normal ischemic preconditioning (NIPC) groups; and iv) hypercholesterolemic PNU282987 postconditioning (HPNU) and normal PNU282987 postconditioning (NPNU) groups. Serum lactate dehydrogenase (LDH), creatine kinase isoenzyme MB (CK-MB), cardiac troponin I (cTnI), tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) levels after ischemia/reperfusion were assayed. Furthermore, infarct size and expression levels of Akt, phosphorylated (p)-Akt and endothelial nitric oxide synthase (eNOS) in ischemic myocardium were assessed. Compared with the NI group, serum LDH, CK-MB, cTnI, TNF-α and IL-6 levels and infarct size were significantly decreased, and myocardial p-Akt/Akt and eNOS/GAPDH ratios were significantly increased in the NIPC and NPNU groups. Compared with the HI group, serum CK-MB, cTnI, TNF-α and IL-6 levels and infarct size were significantly decreased in the HIPC group; however, myocardial p-Akt/Akt and eNOS/GAPDH ratios did not significantly change in the HIPC group. Furthermore, there were no significant difference between the HI and HPNU groups in serum LDH, CK-MB, cTnI, TNF-α and IL-6 levels, infarct size, myocardial p-Akt/Akt and eNOS/GAPDH ratios. In conclusion, hypercholesterolemia could aggravate myocardial ischemia/reperfusion injury, attenuate cardioprotection of ischemic preconditioning and eliminate cardioprotection from α7nAChR agonist postconditioning by enhancing inflammation and inhibiting PI3K/Akt/eNOS pathway.

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Abbreviations: CK-MB, creatine kinase isoenzyme MB; cTnI, cardiac troponin I; eNOS, endothelial nitric oxide synthase; HC, hypercholesterolemic control; HI, hypercholesterolemic ischemia/reperfusion; HIPC, hypercholesterolemic ischemic preconditioning; HPNU, hypercholesterolemic PNU282987 postconditioning; IL-6, interleukin-6; IPC, ischemic preconditioning; IRI, ischemia/reperfusion injury; LAD, left anterior descending coronary artery; LDH, lactate dehydrogenase; LDL, low-density lipoprotein; NC, normal control; NI, normal ischemia/reperfusion; NIPC, normal ischemic preconditioning; NPNU, normal PNU282987 postconditioning; TC, total cholesterol; TG, triglyceride; TNF-α, tumor necrosis factor α; α7nAChR, α7 nicotinic acetylcholine receptor

Key words: hypercholesterolemia, ischemia/reperfusion injury, ischemic preconditioning, α7 nicotinic acetylcholine receptor agonist post-conditioning, cardioprotection

Introduction

The available evidence indicates that a number of interventions can produce a definite protection against myocardial ischemia/reperfusion injury (IRI) (1). Notably, ischemic preconditioning (IPC) can provide a powerful protection against myocardial IRI and is commonly used as a gold standard for evaluating the cardioprotective effect of interventions in experimental studies (2). However, clinical application of IPC is significantly hindered by ethical issues, including a requirement of direct interventions on blood vessels of heart and unpredictability of ischemic heart attack. Thus, it is generally considered that postconditioning is the most valuable treatment of myocardial IRI in clinical practice, especially pharmacological postconditioning (3). In available

literatures, numerous drugs including α7 nicotinic acetylcholine receptor (α7nAChR) agonists, anesthetics, opioid drugs, rosuvastatin, atorvastatin, dexmedetomidine, endomorphin-1, phosphodiesterase and caspase inhibitors have been used for pharmacological postconditioning and have been demonstrated to produce moderate protection against myocardial IRI in normal rats (3,4).

It has been demonstrated that common comorbidities of patients with ischemic heart diseases, such as hypercholesterolemia, hypertension, myocardial hypertrophy, diabetes, obesity and sensory neuropathy, can significantly affect the cardioprotective effectiveness of various interventions including IPC and ischemia postconditioning by different mechanisms (5,6). Therefore, therapeutic interventions cannot be used practically in a clinical setting until their cardioprotection has been demonstrated in the presence of common comorbidities of ischemic heart diseases (7). Because hypercholesterolemia is one of most prevalent comorbidities of ischemic heart diseases, there have been numerous experimental and clinical studies assessing its effect on the cardioprotective potentials of drugs or ischemic preconditioning and postconditioning (8,9). Indeed, the majority of studies indicate that hypercholesterolemia can abolish or attenuate the cardioprotection of IPC (10,11). However, other studies instead indicate that IPC can still preserve cardioprotective potential in hypercholesterolemic animals (12,13). Notably, the detailed mechanisms of how hypercholesterolemia affects cardioprotective effect of IPC have yet to be fully revealed. Moreover, to the best of our knowledge, there has been no study evaluating the effect of hypercholesterolemia on the cardioprotection of postconditioning with α7nAChR agonists. Thus, the present study was designed to compare the cardioprotective effects, inflammatory responses and changes of the PI3K/Akt/endothelial nitric oxide synthase (eNOS) signaling pathway between normal and hypercholesterolemic rats receiving the IPC and α7nAChR agonist postconditioning. The main aims of the present study were to determine the effect of hypercholesterolemia on the cardioprotective efficacy of IPC and α7nAChR agonist postconditioning and to explore the potential mechanisms through which hypercholesterolemia affected their cardioprotection.

Materials and methods

Laboratory animals. The present experiment used 80 SPF-grade male Sprague Dawley rats, aged ~1-month-old and weighing 130-150 g. All animals were supplied by Beijing Vital River Laboratory Animal Technology Co., Ltd. The rats were kept under controlled environmental conditions at a temperature of 20±2˚C, a relative humidity of 60±5% and a 12 h light dark cycle with free access to water and food. After the protocol was approved by the Animal Care and Use Committee of Plastic Surgery Hospital, Chinese Academy of Medical Sciences [approval no. 2017(38); June 16, 2017; Beijing, China], this experiment was conducted in accordance with our institutional guidelines on the use of live animals for research.

Establishment of hypercholesterolemic rat model. As described in previous literature (14), the rats were fed with a high-cholesterol diet containing 2% cholesterol and 0.5% bile salts for 8 weeks. The control rats were fed with a normal diet for 8 weeks. Subsequently, serum total cholesterol (TC), triglyceride (TG) and low-density lipoprotein (LDL) were measured using an AU480 Chemistry Analyzer (Beckman Coulter, Inc.).

Establishment of myocardial IRI rat model. According to the method reported in our previous work (4), a rat model of myocardial IRI was performed. Briefly, the rat was anesthetized with an intraperitoneal injection of 10% chloral hydrate 350 mg/kg, and anesthetic was supplemented during the experiment if needed. After the left thoracotomy and pericardiotomy, myocardial ischemia was achieved by occlusion of the left anterior descending coronary artery (LAD) with a 5-0 silk ligature. Successful occlusion of the LAD was confirmed by the presence of ST segment elevation on electrocardiogram (ECG) and a change in epicardial color from fresh-red to dark-red or paleness of the myocardium. After the ligature was released, adequate myocardial reperfusion of blood flow was verified using epicardial hyperemia and reversion of ECG changes, such as ST segment level in the reperfusion phase descended >50% of ST segment in the ischemia period.

After the experiment was completed, the rats' abdominal cavities were opened to determine whether intraperitoneal injection of drug caused visceral injury or peritonitis. If so, the animal was excluded from data analysis.

Experimental protocols. Using computer-generated random numbers 40 hypercholesterolemic rats and 40 normal rats were randomly divided into four groups (n=10 per group) and received the following different treatments and controls: i) Hypercholesterolemic control (HC) and normal control (NC) groups; ii) hypercholesterolemic ischemia/reperfusion (HI) and normal ischemia/reperfusion (NI) groups; iii) hypercholesterolemic ischemic preconditioning (HIPC) and normal ischemic preconditioning (NIPC) groups; and iv) hypercholesterolemic PNU282987 postconditioning (HPNU) and normal PNU282987 postconditioning (NPNU) groups.

In the HC and NC groups, animals were only subjected to surgical manipulation without ischemia/reperfusion interventions. In the other groups, animals received ischemia/reperfusion interventions including a LAD occlusion for 30 min, followed by 120 min of reperfusion. In the HIPC and NIPC groups, rats were first subjected to the classic IPC interventions before ischemia/reperfusion interventions, namely 5 min of ischemia followed by 5 min of reperfusion for three cycles. Apart from the HPNU and NPNU groups, all animals were injected intravenously with 1 ml normal saline at the end of a 30-min ischemia. In the HPNU and NPNU groups, a highly selective α7nAChR agonist, PNU282987 (cat. no. 123464-89-1; Tocris Bioscience), was intravenously injected immediately before a 120-min reperfusion. According to our previous work (4), the dosage of PNU282987 used for pharmacological postconditioning was 2.0 mg/kg, and it was diluted with 1 ml normal saline immediately before use.

At 120 min of reperfusion, sodium pentobarbital 25 mg/kg was intravenously administered to increase the depth of anesthesia and then a 3-ml blood sample was collected in a tube containing EDTA from the right carotid artery. After settling for 30 min, blood samples were centrifuged at 377.325 x g for

10 min at 4˚C. The supernatants were collected and stored at -80˚C until future analysis. The serum concentrations of TC, TG, LDL, creatine kinase isoenzyme MB (CK-MB) and cardiac troponin I (cTnI) were assessed by using an AU480 Chemistry Analyzer (Beckman Coulter, Inc.). The serum concentrations of tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) concentrations were assessed using the enzyme-linked immunosorbent assay (ELISA) kits (rat TNF-α and IL-6; cat. nos. ab236712, and ab234570, respectively; both Abcam) specific for rat factors, following the manufacturer's instructions (MULTISKAN MK3; Thermo Fisher Scientific, Inc.).

Evaluation of infarct size. After a reperfusion period of 120 min, five anesthetized rats from each group were randomly selected. According to the method reported in our previous work (4), the LAD was reoccluded and 1 ml of 2% Evans blue dye was injected by the carotid artery. When the body was stained blue, the rat was deeply anesthetized with intravenous injection of sodium pentobarbital 100 mg/kg and then was euthanized by intravenous injection of 10% potassium chloride 100 mg/kg. Subsequently, the entire heart was excised, rinsed of excess blue dye and the right ventricle and right and left atria trimmed off. The remaining left ventricle was deep frozen at -20˚C. Subsequently, the frozen left ventricle was cut into ~five slices with 1 mm-thickness from apex to base and all tissue slices were incubated in a 1% solution of 2,3,5-triphenyltetrazolium chloride for 15 min at 37˚C. The infracted tissue stained a characteristic white color, whereas the viable tissue stained red. After overnight fixation at 4˚C in 10% formaldehyde, images of the slices were digitally captured. The slices were analyzed using the Adobe Photoshop CS6 (Adobe Systems, Inc.) by a blinded investigator who assessed the area at risk (AAR) and the infarct size, which was expressed as a percentage of the AAR.

Myocardial expressions of Akt, phosphorylated (p)-Akt and eNOS by western blotting. After a reperfusion period of 120 min, the remaining five rats in each group were deeply anesthetized with intravenous injection of sodium pentobarbital 100 mg/kg and then were euthanized with intravenous injection of 10% potassium chloride 100 mg/kg. The left ventricle was quickly removed and the myocardial tissues from the ischemic area were cut into small pieces of the same weight and stored at -80˚C. The proteins were extracted from myocardial tissue by suspension in radioimmunoprecipitation assay lysis buffer 9 (Beijing BLKW Biotechnology Co., Ltd.). Samples were centrifuged at 28,341.3 x g at 4˚C for 20 min. The protein concentration was measured using bicinchoninic acid assay. An equal amount of protein (30 μg per well) in each group was electrophoresed (SDS-PAGE, 10% of separation gel and 5% of concentration gel) and transferred to a polyvinylidene fluoride membrane. After blocking (5% skimmed milk at room temperature for 2 h) and eluting, the membranes were incubated overnight shaking at a 4˚C condition with monoclonal antibodies against AKT (1:1,000, 4685S; Cell Signaling Technology, Inc.), p-AKT (1:2,000, 4060S; Cell Signaling Technology, Inc.), eNOS (1:1,000, 32027s; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.), respectively. Then, the membranes were washed with Tris-buffered saline with 0.1% Tween solution and incubated with a horseradish peroxidase-conjugated second antibody (1:10,000, goat anti-rabbit immunoglobulin G, 111-035-003, Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. The antigen-antibody complexes in the membranes were visualized using enhanced chemiluminescence and films were exposed in the darkroom. The times of exposure, development and fixing were dependent on the darkness of bands. The films were scanned and saved as TIF image files. The band intensity was quantified using Gel Image system version 4.00 Analysis software (Tanon Science and Technology Co., Ltd.). Finally, expression levels of proteins were acquired by standardizing the grey levels of Akt, p-Akt and eNOS with GAPDH.

Statistical analysis. The primary endpoint of this experiment was infarct size. According to our previous study (15), infarct size was 71.6±8.7 and 36.0±12.5% in the normal rats receiving ischemia/reperfusion and IPC, respectively. Sample size calculation indicated that a sample size of at least 4 rats/group would be required, with a power of 80% and P-value of 0.05. More than 5 rats per group for each observed variable were included in the experiment so as to ensure enough data to fit the ANOVA models and to allow for comparisons among other outcome variables of interest. Statistical analysis of data was performed using SPSS (version 18.0; SPSS, Inc.). For continuous variables, the normal distribution test and Levene test were employed to test the normal distribution and the homogeneity of variance. If the data were normally distributed and had homogeneous variance, they were expressed as mean ± standard deviation. The comparisons of serum TC, LDL and TG levels between NC and HC groups were performed by the unpaired t-test. The comparisons of serum myocardial injury biomarker and inflammatory factor levels, infarct sizes and myocardial Akt and eNOS expression levels among groups were performed using a two- or three-way analysis of variance, as needed. Sidak's test was used for post-hoc multiple comparisons. When data were not normally distributed or had inhomogeneous variance, they were expressed as median (interquartile range). The non-parametric test was employed for statistical analysis of data. The Mann-Whitney U test was used for comparison between groups, and the Kruskal-Wallis test was used for comparisons among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

High cholesterol feed significantly increases serum TC and LDL levels in rats. The serum TC and LDL levels were significantly higher in the HC group compared with the NC group ($P<0.05$); however, serum TG level was not significantly different between the HC and NC groups (Fig. 1). This suggested that a rat model of hypercholesterolemia was successfully established.

Effects of IPC and PNU282987 postconditioning decreases elevation of myocardial injury biomarkers, which are attenuated or eliminated by hypercholesterolemia. In the normal rats, serum LDH, CK-MB and cTnI levels were significantly elevated in the NI, NIPC and NPNU groups compared

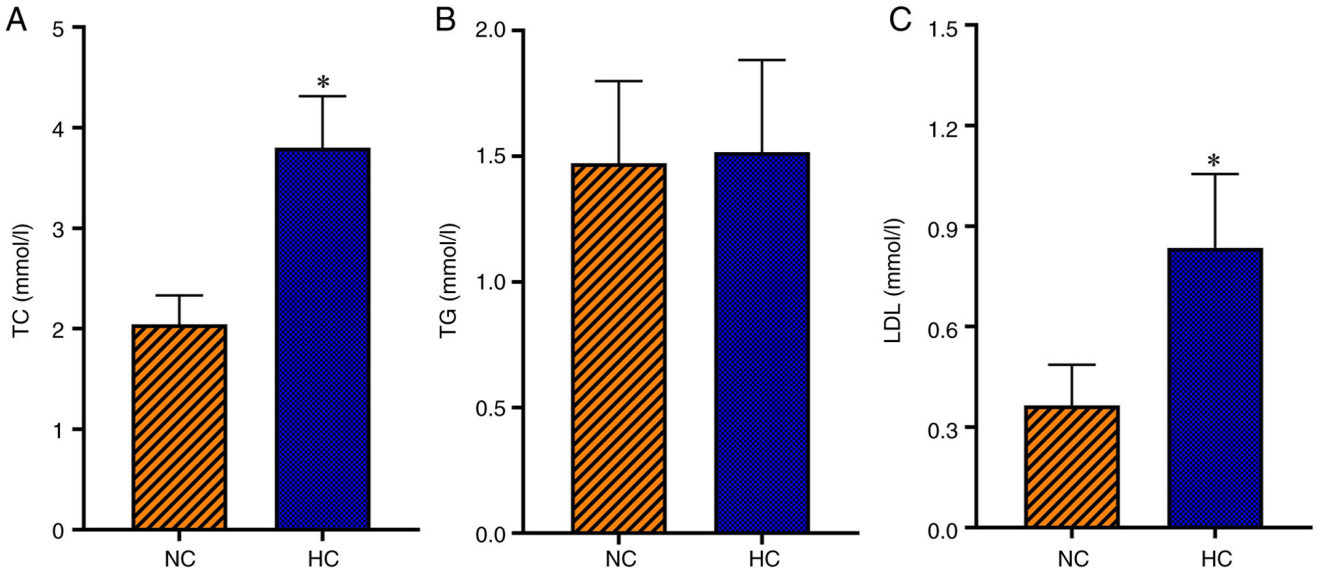

Figure 1. Comparisons of (A) serum TC, (B) TG and (C) LDL levels between normal and hypercholesterolemic rats. *P<0.05 compared with the NC group. TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein.

with the NC group (P<0.05). Whereas serum LDH, CK-MB and cTnI levels were significantly decreased in the NIPC and NPNU groups compared with the NI group (P<0.05). Moreover, serum CK-MB and cTnI levels were significantly elevated in the NPNU group compared with the NIPC group (P<0.05) (Fig. 2).

In the hypercholesterolemic rats, serum LDH, CK-MB and cTnI levels were significantly increased in the HI, HIPC and HPNU groups compared with the HC group (P<0.05); serum LDH, CK-MB and cTnI levels were significantly reduced in the HIPC and HPNU groups compared with the HI group (P<0.05). In addition, serum CK-MB and cTnI levels were significantly elevated in the HPNU group compared with the HIPC group (P<0.05). Compared with the normal rats, serum LDH, CK-MB and cTnI levels were significantly elevated in the corresponding treatment groups of hypercholesterolemic rats (HI vs. NC, HIPC vs. NIPC and HPNU vs. NPNU groups; P<0.05) (Fig. 2).

Infarct size-limiting effect of IPC are attenuated and effect of PNU282987 postconditioning is eliminated by hypercholesterolemia. No myocardial infarction was observed in the HC and NC groups. In the normal rats, infarct size was significantly reduced in the NIPC and NPNU groups compared with the NI group (P<0.05). In addition, infarct size was significantly increased in the NPNU group compared with the NIPC group (P<0.05). In hypercholesterolemic rats, infarct size was evidently reduced in the HIPC group compared with the HI group (P<0.05), but was not significantly changed in the HPNU group (P>0.05). Moreover, infarct size was significantly increased in the HPNU group compared with the HIPC group (P<0.05). Compared with normal rats, infarct size was significantly increased in the corresponding treatment groups of hypercholesterolemic rats (NI vs. HI, NIPC vs. HIPC and NPNU vs. HPNU groups; P<0.05) (Fig. 3). These results indicated that both IPC and PNU282987 postconditioning provided a protection against myocardial IRI in the normal rats, but this cardioprotective effect of IPC was attenuated and that of PNU282987 postconditioning was eliminated in the hypercholesterolemic rats.

Inhibitive effects of IPC and PNU282987 postconditioning on inflammation responses by myocardial ischemia/reperfusion are attenuated or eliminated by hypercholesterolemia. In the normal rats, serum TNF-α and IL-6 levels were significantly increased in the NI, NIPC and NPNU groups compared with the NC group (P<0.05); whereas these levels were significantly reduced in the NIPC and NPNU groups compared with the NI group (P<0.05). Serum TNF-α and IL-6 levels were significantly increased in the NPNU group compared with the NIPC group (P<0.05) (Fig. 4).

In the hypercholesterolemic rats, serum TNF-α and IL-6 levels were significantly increased in the HI, HIPC and HPNU groups compared with the HC group (P<0.05); whereas serum TNF-α and IL-6 levels were significantly reduced in the HIPC group compared with the HI group (P<0.05), but did not significantly change in the HPNU group (P>0.05). Moreover, compared with the HIPC group, serum TNF-α and IL-6 levels were significantly increased in the HPNU group (P<0.05) (Fig. 4).

There were no significant differences in serum TNF-α and IL-6 levels between the NC and HC groups (P>0.05). However, compared with the normal rats, serum TNF-α and IL-6 levels were significantly increased in the corresponding treatment groups of hypercholesterolemic rats (NI vs. HI, NIPC vs. HIPC and NPNU vs. HPNU groups, P<0.05) (Fig. 4). These results indicated that both IPC and PNU282987 postconditioning could inhibit inflammatory responses by myocardial IRI in normal animals, but hypercholesterolemia significantly attenuated anti-inflammatory effect of IPC and eliminated the effect of PNU282987 postconditioning on inflammatory responses.

IPC and PNU282987 postconditioning enhances myocardial Akt phosphorylation and eNOS expression, which are attenuated or eliminated by hypercholesterolemia.

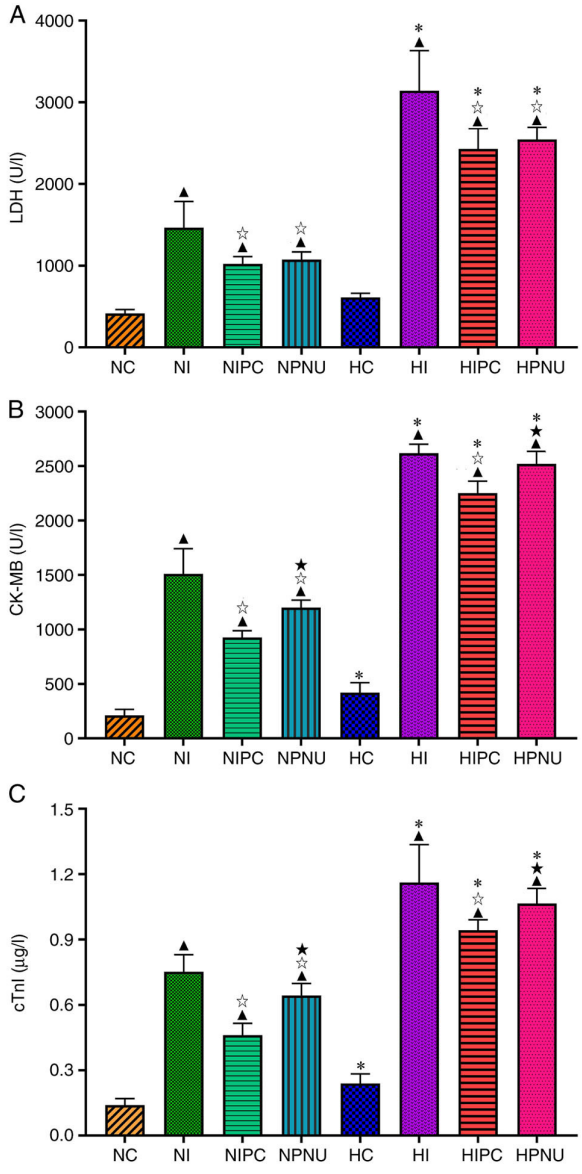

Figure 2. Comparisons of serum (A) LDH, (B) CK-MB and (C) cTnI levels between normal and hypercholesterolemic rats. Intra-group comparisons of normal and hypercholesterolemic rats: ▲P<0.05 compared with the NC or HC group; ☆P<0.05 compared with the NI or HI group; ★P<0.05 compared with the NIPC or HIPC group. Inter-group comparisons between corresponding treatment groups of normal and hypercholesterolemic rats; namely, *P<0.05, HC vs. NC, HI vs. NI, HIPC vs. NIPC and HPNU vs. NPNU groups. LDH, lactate dehydrogenase; CK-MB, creatine kinase isoenzyme MB; cTnI, cardiac troponin I; NC, normal control; NI, normal ischemia/reperfusion; NIPC, normal ischemic preconditioning; NPNU, normal PNU282987 postconditioning; HC, hypercholesterolemic control; HI, hypercholesterolemic ischemia/reperfusion; HIPC, hypercholesterolemic ischemic preconditioning; HPNU, hypercholesterolemic PNU282987 postconditioning.

In the normal rats, myocardial p-Akt/Akt and eNOS/GAPDH ratios were significantly higher in the NI, NIPC and NPNU groups compared with the NC group (P<0.05); these ratios were also significantly higher in the NIPC and NPNU groups compared with the NI group (P<0.05). Moreover, the myocardial p-Akt/Akt ratio was significantly increased in the NPNU group compared with the NIPC group (P<0.05) (Fig. 5).

In the hypercholesterolemic rats, myocardial p-Akt/Akt and eNOS/GAPDH ratios were significantly higher in the HI, HIPC and HPNU groups compared with the HC group (P<0.05). However, compared with the HI group, myocardial p-Akt/Akt and eNOS/GAPDH ratios did not significantly change in the HIPC and HPNU groups (P>0.05). Furthermore, there were no significant differences in the myocardial p-Akt/Akt and eNOS/GAPDH ratios between the HIPC and HPNU groups (Fig. 5).

The myocardial p-Akt/Akt and eNOS/GAPDH ratios were not obviously different between the NC and HC groups (P>0.05). However, myocardial p-Akt/Akt and eNOS/GAPDH ratios were significantly reduced in corresponding treatment groups of hypercholesterolemic rats compared with the normal rats (NI vs. HI, NIPC vs. HIPC and NPNU vs. HPNU groups; P<0.05) (Fig. 5). These results indicated that both Akt and eNOS were involved in the cardioprotective effects of IPC and PNU282987 postconditioning against IRI in normal and hypercholesterolemic rats.

Discussion

In the present study, after 1-month-old SD rats were fed with a high-cholesterol diet for 8 weeks their serum TC and LDL levels significantly increased, which indicated that a hypercholesterolemic rat model had been successfully established (16). Serum LDH, CK-MB and cTnI levels were significantly increased in the normal and hypercholesterolemic rats experiencing myocardial ischemia and reperfusion. Furthermore, serum LDH, CK-MB and cTnI levels were significantly higher in the hypercholesterolemic rats compared with the normal rats (HI vs. NI groups), suggesting that myocardial IRI was more serious in hypercholesterolemic rats. These results were consistent with the findings of previous studies (14,17), in which hypercholesterolemia can aggravate myocardial IRI.

Furthermore, the present study demonstrated that in the normal rats, both IPC and PNU282987 postconditioning significantly decreased serum LDH, CK-MB and cTnI levels, especially the IPC (NIPC and NPNU vs. NI groups, respectively). These findings correspond with results of a previous study (18). All of these findings in the normal rats indicated that both IPC and α7nAChR agonist postconditioning could provide significant protection against myocardial IRI and the cardioprotection of IPC was stronger.

The main aim of the present study was to determine effects of hypercholesterolemia on the cardioprotective efficacy of IPC and α7nAChR agonist postconditioning. The results demonstrated that the IPC significantly reduced serum LDH, CK-MB and cTnI levels in the hypercholesterolemic rats, but the potency of IPC to decrease these biomarkers was significantly weakened in the hypercholesterolemic rats compared with the normal rats. These results indicated that hypercholesterolemia attenuated the protection of IPC against myocardial IRI. This is in accordance with the findings of Ueda *et al* (10) where the cardioprotective effect of IPC is decreased in hypercholesterolemic rabbit hearts subjected to ischemia/reperfusion.

To the best of our knowledge, there has been no study assessing the effect of hypercholesterolemia on cardioprotection of α7nAChR agonist postconditioning. The present experiment indicated that serum LDH, CK-MB and cTnI levels

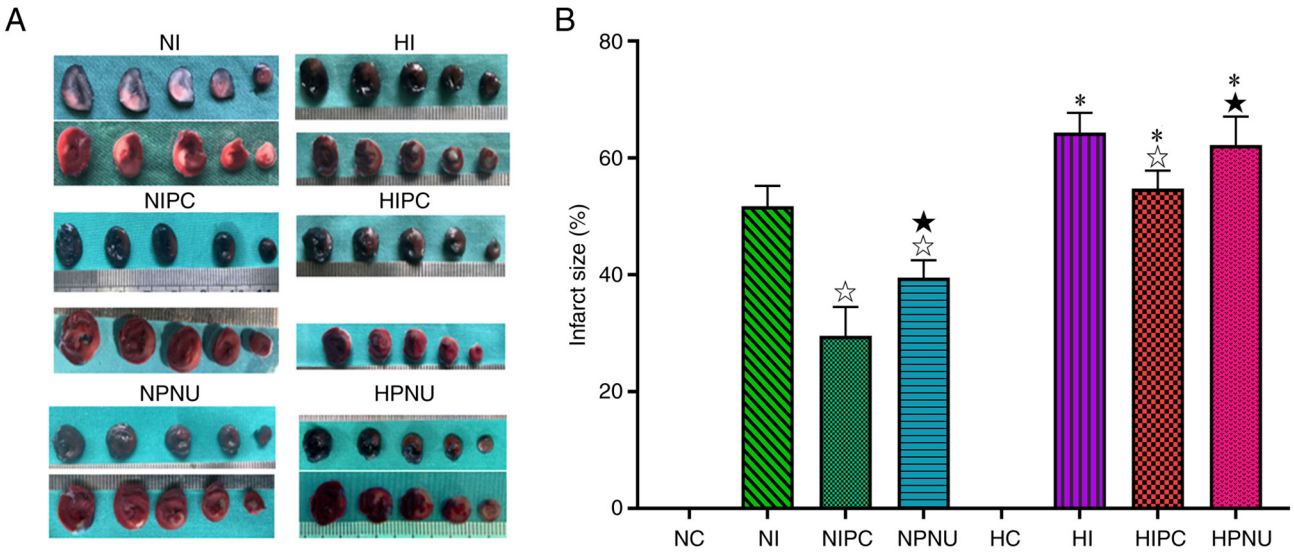

Figure 3. Representative images of (A) Evan's blue staining and TTC staining of cardiac slices in normal and hypercholesterolemic rats and (B) comparison of myocardial infarct size (%). Intra-group comparisons of normal and hypercholesterolemic rats: ☆P<0.05 compared with the NI or HI group: ★P<0.05 compared with the NIPC or HIPC group. Inter-group comparisons between corresponding treatment groups of normal and hypercholesterolemic rats: *P<0.05, HI vs. NI, HIPC vs. NIPC and HPNU vs. NPNU groups. LDH, lactate dehydrogenase; CK-MB, creatine kinase isoenzyme MB; cTnI, cardiac troponin I; NC, normal control; NI, normal ischemia/reperfusion; NIPC, normal ischemic preconditioning; NPNU, normal PNU282987 postconditioning; HC, hypercholesterolemic control; HI, hypercholesterolemic ischemia/reperfusion; HIPC, hypercholesterolemic ischemic preconditioning; HPNU, hypercholesterolemic PNU282987 postconditioning.

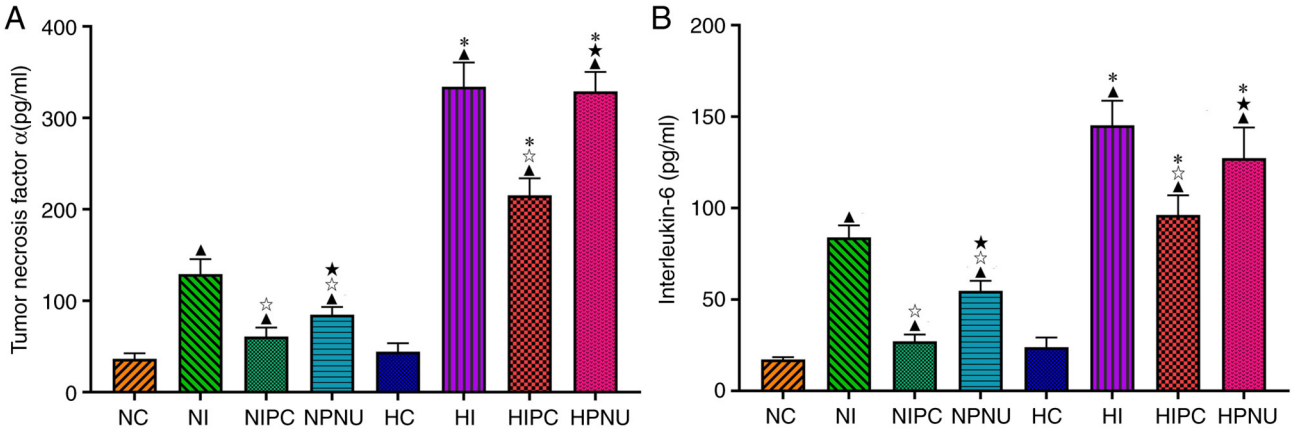

Figure 4. Comparisons of serum (A) IL-6 and (B) TNF-α levels between normal and hypercholesterolemic rats. Intra-group comparisons of normal and hypercholesterolemic rats: ▲P<0.05 compared with the NC or HC groups; ☆P<0.05 compared with the NI or HI groups; ★P<0.05 compared with the NIPC or HIPC groups. Inter-group comparisons between corresponding treatment groups of normal and hypercholesterolemic rats: *P<0.05, HC vs. NC, HI vs. NI, HIPC vs. NIPC and HPNU vs. NPNU groups. LDH, lactate dehydrogenase; CK-MB, creatine kinase isoenzyme MB; cTnI, cardiac troponin I; NC, normal control; NI, normal ischemia/reperfusion; NIPC, normal ischemic preconditioning; NPNU, normal PNU282987 postconditioning; HC, hypercholesterolemic control; HI, hypercholesterolemic ischemia/reperfusion; HIPC, hypercholesterolemic ischemic preconditioning; HPNU, hypercholesterolemic PNU282987 postconditioning; IL, interleukin; TNF-α, tumor necrosis factor α.

were not significantly different between the HPNU and HI groups, indicating that hypercholesterolemia eliminated the cardioprotection from α7nAChR agonist postconditioning.

Infarct size is a gold standard parameter that evaluates the severity of myocardial injury and cardioprotective efficacy of interventions in the animal experiment (4). Consistent with the aforementioned changes of myocardial injury biomarkers, the present study revealed that in the normal rats subjected to myocardial IRI, both IPC and PNU282987 postconditioning significantly reduced the infarct size by 42.9 and 23.7% (NIPC and NPNU vs. NI groups), respectively. These results agree with the findings of previous studies (4,18). All of these support the aforementioned conclusions obtained by the myocardial injury biomarkers that the two interventions can produce a significant protection against myocardial IRI in the normal rats, but the cardioprotective potency of IPC is stronger.

However, the infarct size was increased by 19.6% in the hypercholesterolemic rats compared with the normal rats (HI vs. NI groups). This further supports that hypercholesterolemic rats are more vulnerable to myocardial IRI than normal rats. Similarly, in hypercholesterolemic rats the IPC only reduced the infarct size by 14.9% (HIPC vs. HI groups), which was significantly smaller compared with that in the

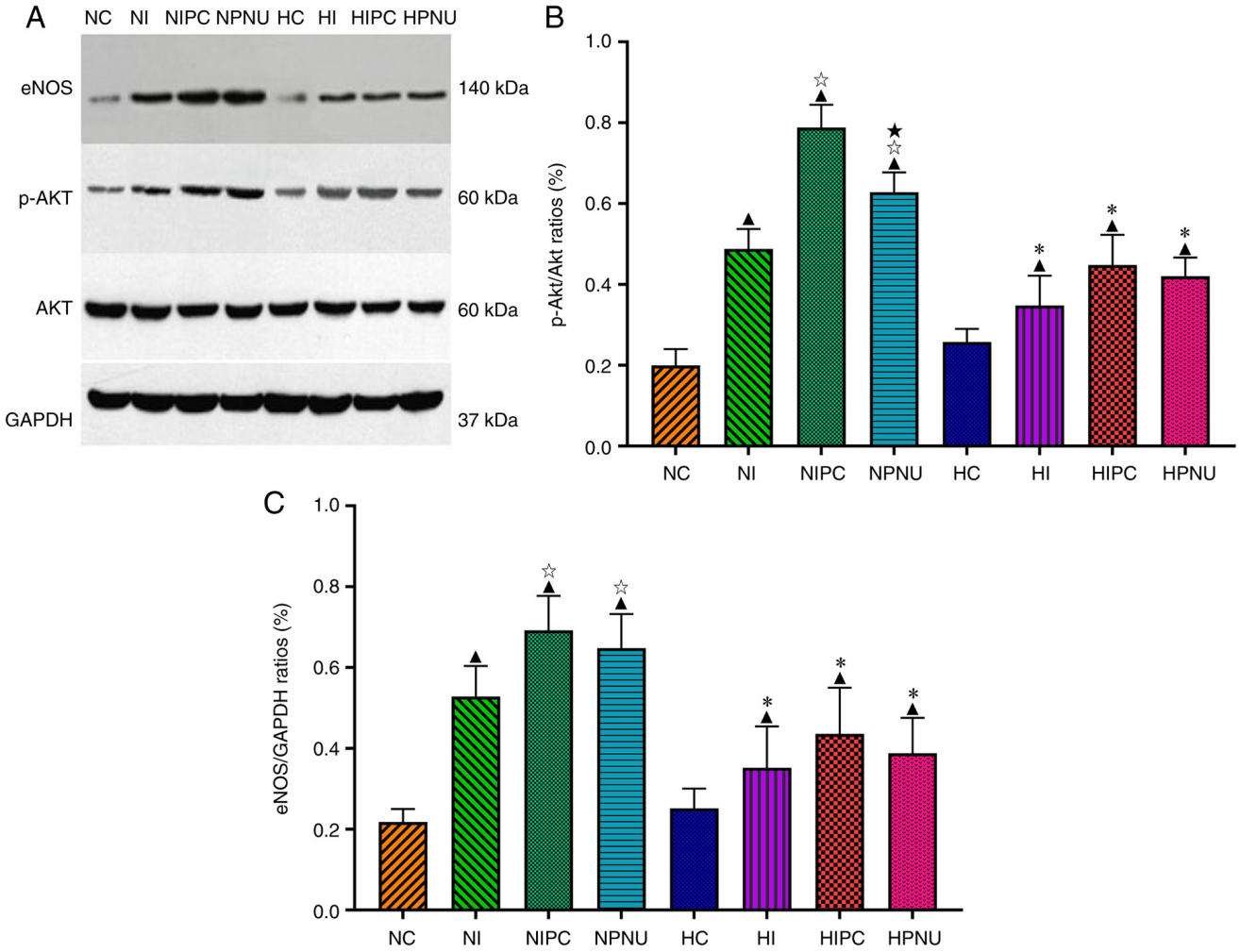

Figure 5. (A) Representative western blotting images of myocardial p-Akt, Akt and eNOS expressions and comparisons of (B) myocardial p-Akt/Akt and (C) eNOS/GAPDH ratios between normal and hypercholesterolemic rats. Intra-group comparisons of normal and hypercholesterolemic rats: ▲P<0.05 compared with the NC or HC group; ☆P<0.05 compared with the NI or HI group; ★P<0.05 compared with the NIPC or HIPC group. Inter-group comparisons between corresponding treatment groups of normal and hypercholesterolemic rats: *P<0.05, HC vs. NC, HI vs. NI, HIPC vs. NIPC and HPNU vs. NPNU. LDH, lactate dehydrogenase; CK-MB, creatine kinase isoenzyme MB; cTnI, cardiac troponin I; NC, normal control; NI, normal ischemia/reperfusion; NIPC, normal ischemic preconditioning; NPNU, normal PNU282987 postconditioning; HC, hypercholesterolemic control; HI, hypercholesterolemic ischemia/reperfusion; HIPC, hypercholesterolemic ischemic preconditioning; HPNU, hypercholesterolemic PNU282987 postconditioning; p-, phosphorylated; eNOS, endothelial nitric oxide synthase.

normal rats (NIPC vs. NI groups; 42.9%). This indicated that hypercholesterolemia significantly decreased the infract size-limiting effect of IPC. The present result that hypercholesterolemia attenuated cardioprotection of IPC was in line with the results of Ueda *et al* (10) in the hypercholesterolemic rabbit heart subjected to ischemia/reperfusion and with the results of Ungi *et al* (19) in patients undergoing coronary angioplasty. Moreover, Kocić *et al* (20) demonstrated that hypercholesterolemia completely abolishes the cardioprotective effect of IPC in isolated stunned papillary rat muscle.

However, in the available literature on the effect of hypercholesterolemia on cardioprotection of IPC other studies report different findings. Iliodromitis *et al* (12) demonstrated that IPC preserves its cardioprotection in the myocardial IRI model of hypercholesterolemic rabbits (infract size, 55.2±5.9 and 17.9±4.2% in control and IPC groups, respectively). Furthermore, Jung *et al* (13) confirmed that experimental hypercholesterolemia does not affect the infarct size sparing of IPC in the rabbit heart subjected to ischemia/reperfusion (63±3 and 21±3% in control and IPC groups, respectively). These inconsistent results may be mainly attributable to the differences among various studies in the experimental designs, timings of IPC implementation, durations and cycle numbers of IPC, study objects and methods of making a hypercholesteremic model. For example, in studies by Iliodromitis *et al* (12) and Jung *et al* (13), IPC intervention includes two cycles of 5 min ischemia separated by 10 min reperfusion before the index ischemia. In the present experiment, IPC intervention was performed using the classic scheme, including three cycles of 5 min ischemia followed by 5 min reperfusion before the index ischemia. Notably, hypercholesterolemic rabbit and rat IRI models are applied in both the previous works and this present study, though different animals share various anatomical and physiological characteristics of hearts.

Interestingly, the present results indicated that PNU282987 postconditioning did not significantly reduce the infarct size in the hypercholesterolemic rats (HPNU vs. HI groups),

suggesting that the infarct size-limiting effect of PNU282987 postconditioning is completely abolished by hypercholesterolemia. This supports the above findings from myocardial injury biomarkers.

The available evidence indicates that IRI is a result of complex interactions of multiple pathogenic factors (3). Of them, inflammation is an important pathogenic factor, involving numerous cytokines, adhesion molecules, activation of complement cascade system and toll-like receptors (21). As IL-6 and TNF-α are important cytokines that can accurately reflect the development and severity of inflammatory responses, they are commonly used as the indicators that assess the characteristics of inflammatory responses during myocardial IRI process (22). The present study demonstrated that in the normal rats, both IPC and PNU282987 postconditioning significantly reduced serum IL-6 and TNF-α levels, but the ability of IPC to decrease serum levels of two cytokines was significantly stronger compared with that of PNU282987 postconditioning. In available literature, inhibition of two interventions on inflammatory responses induced by myocardial IRI has been considered as a notable mechanisms for their cardioprotection (4,15). However, the present experiment demonstrated that serum IL-6 and TNF-α levels in the HIPC group were significantly lower compared with those in the HI group, but were higher compared with those in the NIPC group. These results suggested that the inhibitory effect of IPC on the inflammatory responses induced by myocardial IRI were significantly weakened in the presence of hypercholesterolemia. In addition, serum IL-6 and TNF-α levels were not significantly different between the HI and HPNU groups, indicating that hypercholesterolemia completely eliminates the inhibitory effect of PNU282987 postconditioning on inflammatory responses induced by myocardial ischemia/reperfusion. Therefore, it is concluded that hypercholesterolemia can significantly attenuate inhibitive effect of cardioprotective interventions on the inflammatory responses induced by myocardial ischemia/reperfusion. This may be one of the reasons why cardioprotection of the interventions including IPC is decreased in the presence of hypercholesterolemia.

PI3K/Akt is a signaling pathway widely present in cells and is involved in inflammation and cell activation, survival and apoptosis (23). It is generally considered that activation of the PI3K/Akt signaling pathway can protect the myocardium from lethal IRI (24). eNOS, which is continuously expressed in mammalian cardiomyocytes, is a downstream effector of Akt and is regulated by the PI3K/Akt signaling pathway. The available evidence indicates that the PI3K/Akt/eNOS signaling pathway plays an important role in the mechanisms of cardioprotection by various interventions such as delayed preconditioning, dexmedetomidine and baicalin (25-27). It is reported that a specific knock-out of eNOS gene can significantly increase the sensitivity of myocardium to IRI and eliminate the protection of IPC against myocardial IRI (28). Furthermore, hypercholesterolemia can downregulate the expression of eNOS and thus decrease the generation of nitric oxide to induce vascular endothelial dysfunction (29), which may affect the function of coronary artery in the myocardium.

The present study demonstrated that after ischemia/reperfusion, myocardial p-Akt/Akt and eNOS/GAPDH ratios were significantly decreased in the hypercholesterolemic rats compared with the normal rats, suggesting that myocardial Akt phosphorylation and eNOS expression are significantly inhibited in the presence of hypercholesterolemia. Specifically, the present experiment demonstrated that PNU282987 postconditioning enhanced myocardial Akt phosphorylation and eNOS expression, and reduced serum myocardial injury biomarker levels and infarct size in the normal rats, but it did not lead to significant changes in the infarct size, Akt phosphorylation and eNOS expression in the hypercholesterolemic rats. Based on these findings, it is hypothesized that hypercholesterolemia abolishes the cardioprotection of α7nAChR agonist postconditioning by eliminating inflammatory inhibition and inhibiting activation of PI3K/Akt/eNOS signaling pathway. In the hypercholesterolemic rabbit heart subjected to ischemia/reperfusion, Ueda *et al* (10) demonstrated that pravastatin can restore the cardioprotective effect of IPC by activating ecto-5'-nucleotidase. Thus, the present study considered that both restoring regulation of the cholinergic anti-inflammatory pathway on inflammatory responses and provoking activation of myocardial PI3K/Akt/eNOS signaling pathway may be feasible strategies to improve the cardioprotection of α7nAChR agonist postconditioning in the presence of hypercholesterolemia (7). However, these results deserve further studies.

The present study indicated that in hypercholesterolemic rats, IPC did not significantly enhance activation of the PI3K/Akt/eNOS signaling pathway in the ischemic myocardium, and inhibition of IPC on the inflammatory responses induced by myocardial ischemia/reperfusion was significantly weakened. However, notably, differing from the result that hypercholesterolemia completely eliminated cardioprotection from PNU282987 postconditioning, the IPC still exerted a certain level of protection against myocardial IRI in the hypercholesterolemic rats, though cardioprotective potency of IPC was significantly weakened. This may be because that beside inhibition of inflammatory responses and activation of the PI3K/Akt/eNOS signaling pathway, cardioprotection of IPC is also attributable to other mechanisms. The available evidence indicates that activation of reperfusion injury salvage kinase pathway, survival activating factor enhancement pathway, Janus activated kinase signal transducer and activator of transcription pathway, 70 ribosomal protein S6 kinase and glycogen synthase kinase 3β, opening of mitochondrial permeability transition pore and ATP-sensitive K^+ channels and inhibition of apoptosis all are involved in the protection of IPC against myocardial IRI (2,7,23,24). Furthermore, it has been indicated that hypercholesterolemia can inhibit the opening of mitochondrial ATP-sensitive K^+ channels in the rabbit heart subjected to ischemia/reperfusion (30). In fact, opening of mitochondrial ATP-sensitive K^+ channels is considered as a major component involved in the cardioprotection of IPC (23). Thus, the detailed roles of these factors in the mechanisms that hypercholesterolemia affects the cardioprotective effectiveness of IPC also deserves further studies.

In summary, the present study demonstrated that hypercholesterolemia could significantly aggravate myocardial IRI, weaken cardioprotection of IPC and eliminate cardioprotection of α7nAChR agonist postconditioning by

enhancing inflammatory responses and inhibiting activation of PI3K/Akt/eNOS signaling pathway. Thus, both enhancing inhibition of inflammatory responses and facilitating activation of the PI3K/Akt/eNOS signaling pathway may be the useful measures to improve cardioprotective efficacy of two interventions in the presence of hypercholesterolemia.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CW and FSX conceived and designed the experiments. CW, YHW, XL and JHJ performed the experiments. CW, XL and FSX analyzed and interpreted the results of the experiments. CW wrote the manuscript. WC and FSX confirm the authenticity of all the raw data. FSX revised manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study protocol was approved by the Animal Care and Use Committee of Plastic Surgery Hospital, Chinese Academy of Medical Sciences [approval no. 2017(38); June 16, 2017; Beijing, China].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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