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In vivo gum arabic-coated tetrahydrobiopterin protects against myocardial ischemia reperfusion injury by preserving eNOS coupling

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ABSTRACT

Aims: Exogenous tetrahydrobiopterin (BH_4), an indispensable cofactor of endothelial nitric oxide synthase (eNOS), supplementation has been proved to be of advantage to improve cardiovascular function. Nevertheless, due to its highly redox-sensitive and easy to be oxidized, there is an urgent need to develop an appropriate BH4 formulation for clinical therapy. Gum Arabic (GA) has been considered as an alternative biopolymer for the stabilization and coating of drugs. The effects of GA on protecting BH_4 from being oxidized were investigated in a rat model of myocardial ischemia-reperfusion (I/R).

Main methods: Rats were subjected to 60-min of *in vivo* left coronary artery occlusion and varying periods of reperfusion with or without pre-ischemic GA-coated BH_4 supplementation (10 mg/kg, oral). Myocardial infarction, fibrotic area and left ventricle ejection fraction were correlated with cardiac BH_4 content, eNOS protein, NOS enzyme activity, and ROS/NO generation.

Key findings: Pretreatment of rats with GA-coated 6R-BH_4, 24 h before myocardial ischemia, resulted in smaller myocardial infarction, improved left ventricular function and inhibited fibrosis, correlated with maintained high levels of cardiac BH_4 content, preserved eNOS activation and dimerization, and decreased ROS generation. However in uncoated group, 6R-BH_4 treatment did not reduce acute and chronic myocardial I/R injury compared with control I/R rats, which was closely related with the marked loss of myocardial BH4 levels during I/R.

Significance: These findings provide evidence that *in vivo* pre-ischemic oral GA-coated BH_4 administration preserves eNOS function secondary to maintaining cardiac BH_4 content, and confers cardioprotection after I/R.

1. Introduction

Tetrahydrobiopterin (BH_4), originally identified as an indispensable cofactor for the activity of nitric oxide synthases (NOSs) [1,2] with dimeric formation, was reported to be concerned with NO synthesis [3], which has multiple actions in the cardiovascular system such as maintenance of blood pressure, regulation the force and rate of heart contraction, widening blood vessels and increasing blood flow [4,5]. When BH_4 bioavailability is low because of oxidation or insufficient bio-synthesis *in vivo*, the ferric heme-NO complex can't be reduced to the ferrous heme-NO species in NOS, resulting in NOS dissociation and superoxide ($\cdot O2^-$) release, a process termed "NOS uncoupling" [6]. This ability for NOS to generate reactive oxygen species (ROS), such as $\cdot O2^-$, instead of NO is considered to be related with vascular dysfunction. Furthermore, it's important to sustain sufficient BH_4 levels *in vivo* for full NOS functionality.

Endothelial NOS (eNOS) is the major source of NO production in endocardial endothelial cells and cardiomyocytes [7]. Ischemia/

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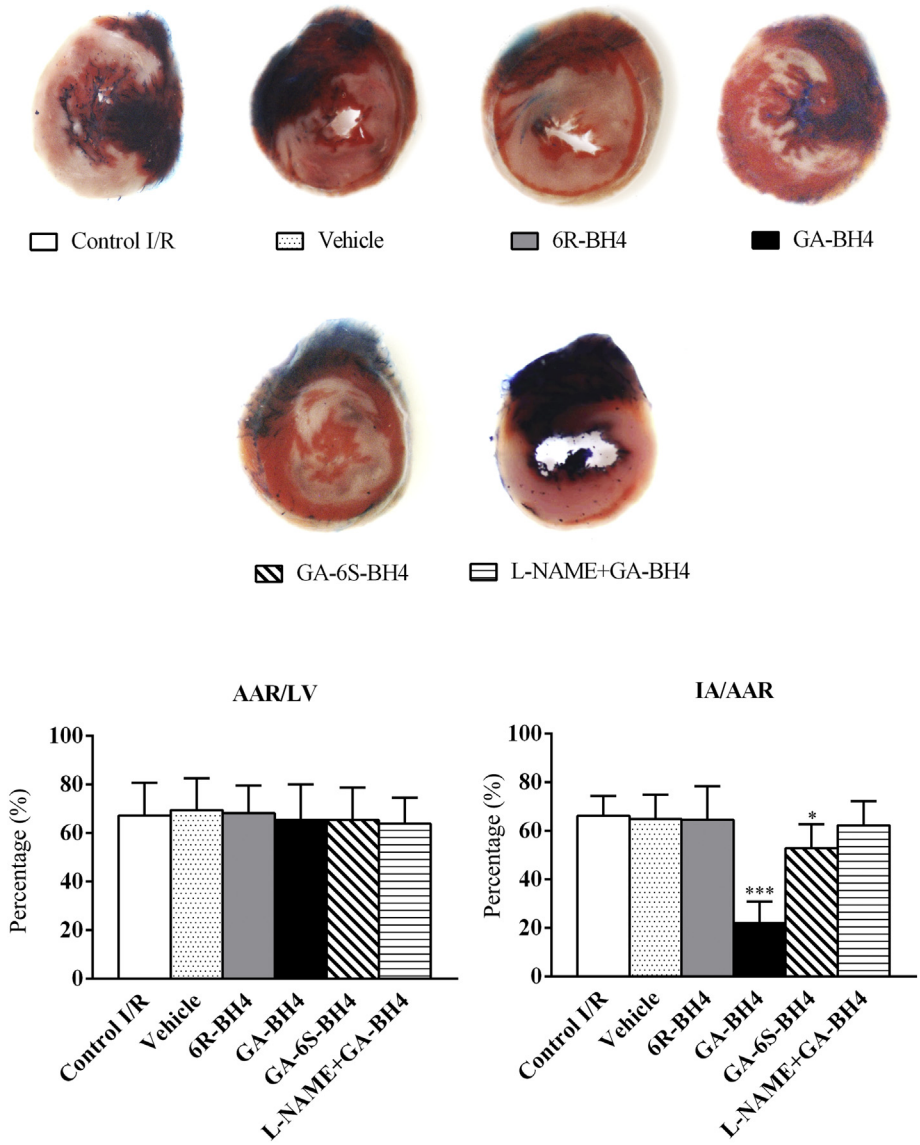

Fig. 1. Myocardial infarction in rat hearts subjected to 60-min LCA ligation and 16-hour reperfusion with and without vehicle, 6R-BH4, GA-BH4, GA-6S-BH4 or NOS inhibitor L-NAME. Representative sections of the hearts stained with Evan's blue and TTC after ischemia-reperfusion (I/R) are shown above the bar graphs. Area at risk (AAR) is expressed as a percentage of left ventricle (LV), AAR/LV. Infarct area (IA) is expressed as a percentage of AAR, IA/AAR. Values are means ± SD. N = 8/group. ***$P < 0.001$, *$P < 0.05$ *vs.* control I/R.

reperfusion (I/R) in hearts is associated with reduced NO and increased ROS generation [8,9] that is concerned with cellular defects involved with both endothelial and myocyte dysfunction [10]. More importantly, with oxidative stress during I/R injury in hearts, intracellular BH_4 can fall below critical level leading to eNOS uncoupling with shift from NO to ROS generation [11,12]. Studies supplementing BH_4, by either genetic or pharmacological means, have shown improvements in cardiovascular function [13–17].

BH_4 is not only highly redox-sensitive and easy to be oxidized [5,18], but also an effective scavenger of ROS [19–21]. Until now a lot of efforts have been made to enhance BH_4 bioavailability by combining with other pharmacological agent such as liposome [13], folic acid, vitamin C or statins [22], however the clinical effects of these combinations are either poor, not related to BH_4 or associated with unavailable technique clinically. Therefore, there is a great need to develop an appropriate BH_4 formulation for clinical therapy.

Gum Arabic (GA) is a complex polysaccharide consisting mainly of potassium, calcium and magnesium salts of polyarabic acid, with residues of rhamnose, galactose, arabinose and glucuronic acid. It is degraded slowly in the colon by bacterial enzymes to achieve effective drug delivery [23]. It is a kind of effective stabilizer, which can form a protective matrix around dispersed compounds, entraps them inside the matrix and prevents them contact with air and oxidation [24]; and GA-coated formulations are informed to improve the stability and efficiency of poorly available drugs [25]. Importantly, its characteristics of solubility, emulsification qualities, low viscosity and excellent retention of volatile compounds make GA primarily used in the food and pharmaceutical industries [26]. Therefore, instead of cosupplementation of BH_4 with another pharmacological agent, we developed a novel BH_4 formulation within GA and demonstrated that this novel GA-coated BH_4 oral administration can rapidly increase BH_4 content and reverse the loss of myocardial eNOS activity during *in vivo* I/R and protect heart against acute and chronic I/R injury.

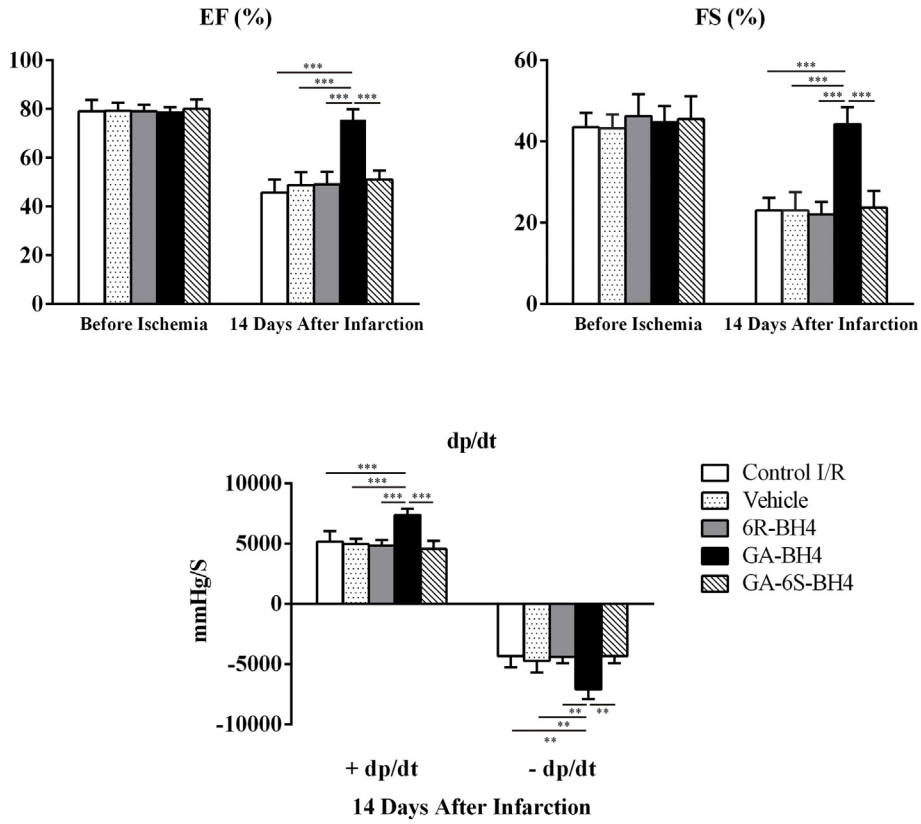

Fig. 2. Hemodynamic parameters assessed by echocardiography and cardiac catheterization 14 days after I/R. Note the improvement of ejection fraction (EF), fractional shortening (FS), and ± dP/dt in the GA-BH_4 group. N = 6/group. ***$P < 0.001$.

2. Methods

2.1. GA coating procedure

GA (5%) was suspended in distilled water at 50 °C and cooled to room temperature. 6R-BH4 (BH4) or 6S-BH4 (Cayman Chemical, USA) was dissolved in GA solution with continuous shaking at 4 °C overnight (70 mg/L). The formulation we made is stored at 4 °C after 50 μm mesh filtering.

2.2. Animals, BH4 dose and treatment protocols

Animal protocols were approved by the Animal Care and Research Committee of Huazhong University of Science and Technology.

Male Sprague-Dawley (SD) rats weighing ~250 g were randomly allocated to different groups. The rats received orally one of five drinking water solutions 24 h before ischemia (BH_4 dose: 10 mg/kg/day): tap water (control group), GA-coated 6R-BH_4 solution (GA-BH_4 group), GA-coated 6S-BH_4 solution (GA-6S-BH_4 group), GA solution only (vehicle group) or 70 mg/L 6R-BH_4 without GA solution (6R-BH_4 group). The effect of NOS inhibition on BH_4-mediated alterations was evaluated by administration of L-NAME (10 mg/kg, iv) 5 min before myocardial ischemia (L-NAME + GA-BH_4 group).

2.3. In vivo myocardial I/R

Myocardial I/R *in vivo* and myocardial infarct size measurements were performed in male SD rats as described previously with slight modification [27]. After 60 min of regional ischemia and defined periods of reperfusion (0 min-, 5 min-, 10 min-, 16 h-, 48 h- and 14 d-), hearts were harvested for either myocardial infarct size or fibrotic area measurements or the area at risk (AAR) from each heart, frozen in liquid nitrogen, and stored at −80 °C until use.

2.4. Infarct size measurement

After 16 h of reperfusion, rats were sacrificed with an overdose of pentobarbital. The heart was excised and 1% solution of triphenyl tetrazolium chloride (Sigma Chemical Co.) was injected into the aorta to visualize the infarct area. Then the coronary branch was reoccluded and Evans blue dye (4%, Sigma Chemical Co.) was injected into the aorta. The AAR was defined as the area without blue staining. The left ventricular (LV) was sectioned into six slices parallel to the atrioventricular ring. Each slice was weighed and photographed. The risk and infarct areas were traced on each LV slice and multiplied by the slice's weight, then expressed as a fraction of the risk area or LV for each heart.

2.5. Echocardiogram

Echocardiograms were recorded with an echocardiographic system equipped with a 7.5-MHz imaging transducer 14 days after myocardial I/R. After cardiac echocardiography, a micromanometer-tipped catheter was inserted into the LV for recording ± dP/dt.

2.6. Fibrotic area measurement

The rats were sacrificed 14 days after I/R. The hearts were removed and cut into six transverse slices, and each slice was fixed with 10% buffered formalin and embedded in paraffin; 4-μm-thick sections were stained with Masson's trichrome. Quantitative assessment of the fibrotic area as a percentage of the LV was performed with the Pannoramic MIDI II scanner system (3DHISTECH Ltd., Hungary).

2.7. Measurement of myocardial BH4 by reverse-phase high-performance liquid chromatography (HPLC)

Myocardial BH4 content was determined by HPLC with

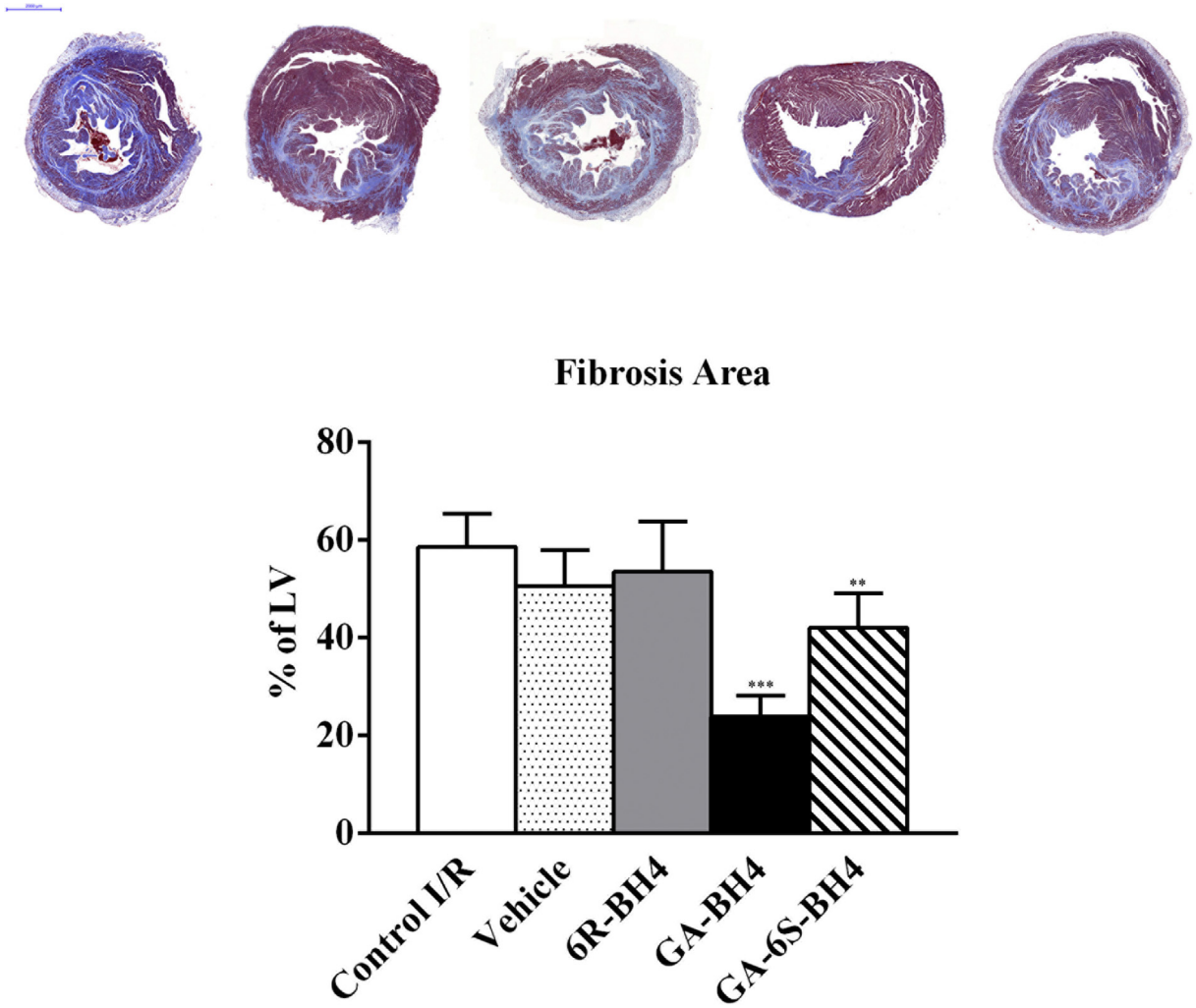

Fig. 3. Fibrosis area determined by Masson's trichrome staining. Photomicrograph of myocardium with Masson's trichrome staining from control I/R, vehicle, 6R-BH_4, GA-BH_4 or GA-6S-BH_4 groups subjected to 60 min of ischemia followed by 14 days of reperfusion (top). Thinning of the infarcted LV wall can be seen. Area stained blue represent fibrotic infiltration (bar = 2000 μm). The fibrosis area is expressed as a percentage of LV (bottom). Values are means ± SD. N = 6/group. ***$P < 0.001$, **$P < 0.01$ *vs.* control I/R.

electrochemical detection as described previously [14]. After defined periods of reperfusion, the heart regions were rapidly frozen in liquid nitrogen, finely ground, and suspended in water containing diethylene triamine pentaacetic acid (DTPA, 0.2 mM), dithiothreitol (DTT, 1 mM) and ascorbic acid (1 mg/ml) to prevent auto-oxidation. HPLC separation was performed and BH4 measured directly by the electrochemical cell at +50 mV.

2.8. eNOS enzyme activity assay

After defined periods of reperfusion, rat heart tissue was homogenized in ice-cold lysis buffer [25 mmol/l Tris-HCl, pH 7.4, 1 mmol/l EDTA, 1 mmol/l ethyleneglycoltetraacetic acid (EGTA)] and spun for 5 min at 10,000*g* and the lysate removed. eNOS activity was determined by the conversion of [3H] L-arginine to [3H] L-citrulline using a NOS activity assay kit (Cayman Chemical, USA). In brief, lysates were incubated in the presence or absence of the specific eNOS inhibitors L-NG-nitroarginine methylester (L-NAME, 100 mol/l) with a reaction buffer and incubated at 37 °C for 60 min. The reaction was then stopped by the addition of ice-cold stopping buffer and equilibrated resin to each sample. [3H] L-citrulline was eluted from the samples and radioactivity quantified in a liquid scintillation counter (PerkinElmer, USA). Protein concentrations of tissue lysates were determined by the BCA protein assay (Beyotime Biotech., China) and retrieved [3H] L-citrulline values were normalized to total protein (pmol/min/mg protein). eNOS activity was determined by subtracting total counts from L-NAME blocked counts.

2.9. eNOS expression detected by immunoblotting

After 5 min of myocardial reperfusion, the AAR of rat heart was homogenized in lysis buffer on ice. Portions (80 μg) of myocardial tissues were subjected to SDS–PAGE. For immunoblot analysis of the dimeric form of eNOS, samples were not heated and the temperature of the gel was maintained below 10 °C during electrophoresis (low-temperature SDS–PAGE). The blots were probed by primary antibody against eNOS and phospho-eNOS (Ser1177) (Cell Signaling Tech, USA). The signal intensity of blots was digitalized and quantified using Image J.

2.10. Measurement of ROS and NO generation in situ

Four rats in each group were sacrificed by intraperitoneal injection of pentobarbital sodium after 5 min of myocardial reperfusion. An 18-gauge needle connected to an infusion pump was inserted in the jugular vein. After the thoracic aorta was cut, the whole heart was perfused

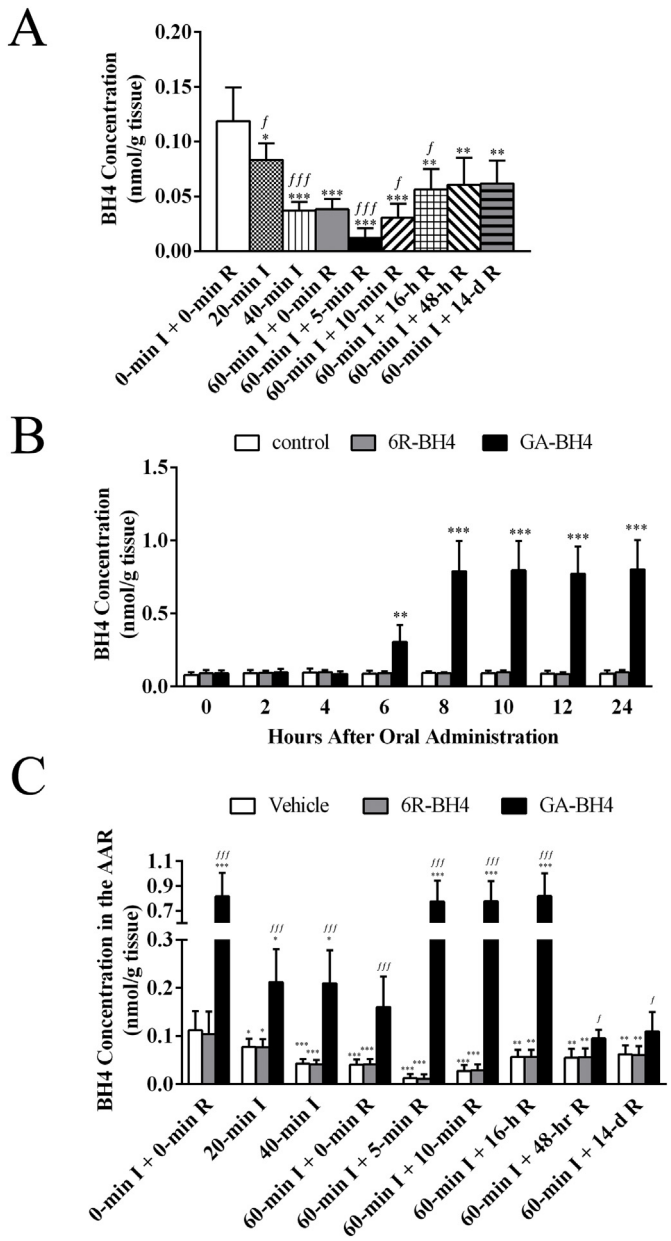

Fig. 4. BH4 concentration in the AAR as determined by HPLC method. A, BH4 levels in the AAR of the heart after I/R. Data are mean ± SD of 6 rats in each group. ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ *vs.* 0-min I + 0-min R (baseline) in the AAR; *fff*$P < 0.001$ and *f*$P < 0.05$ *vs.* previous time point. B, Myocardial BH4 levels after oral GA-coated/uncoated BH_4 administration. Values are means ± SD of 6 rats in each group. ***$P < 0.001$ and **$P < 0.01$ *vs.* time-matched control group. C, BH4 levels in the AAR of the heart during I/R after BH4 treatment. N = 6/group. Values are means ± SD. ***$P < 0.001$ and *$P < 0.05$ *vs.* 0-min I + 0-min R (baseline) in AAR of vehicle group; *fff*$P < 0.001$ and *f*$P < 0.05$ *vs.* time-matched vehicle group.

with 37 °C PBS (flow rate: 5 ml/min). Once blood had been removed, the whole heart was perfused with PBS containing 0.01 mmol/l diaminorhodamine-4M AM (DAR-4M AM; Sigma-Aldrich, USA); 0.05 mmol/l dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, USA); 0.1 mmol/l L-Arg; and 2 mmol/l $CaCl_2$ for an additional 10 min at a flow rate of 5 ml/min [28]. All unreacted DAR-4M AM and DCFH-DA were removed by postperfusion with PBS for 10 min. After fixation with 4% paraformaldehyde perfusion, the hearts were cut distal to left coronary artery suture into 4-μm-thick sections and enveloped on the slide glass. Fluorescent images of ROS and NO were obtained using confocal

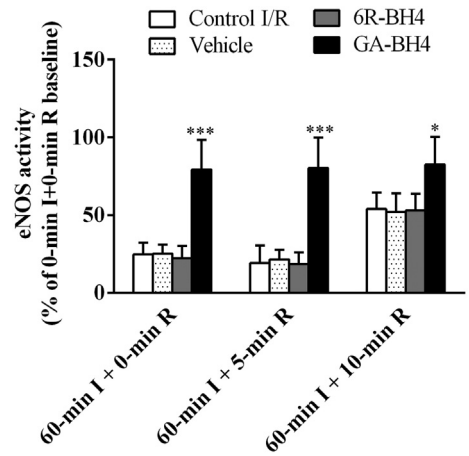

Fig. 5. eNOS activity in the AAR of rat hearts was measured by [3H] L-arginine to [3H] L-citrulline conversion and was determined by subtracting total counts from L-NAME blocked counts. Values are means ± SD expressed as percentage of baseline (0-min I + 0-min R) for eNOS activity. $N = 5$/group. ***$P < 0.001$ and *$P < 0.05$ *vs.* time-matched control I/R group.

laser-scanning microscopy (Leica Microsystems, Germany). The wavelength was as follows: DAR-4M AM, excitation at 560 nm and emission at 575 nm, and DCFH-DA, excitation at 490 nm and emission at 530 nm. Fluorescence intensity was quantified using the software (Leica Microsystems, Germany) and were evaluated in greater than five focal fields in at least four slides per animal.

2.11. Statistical analysis

All results are expressed as the mean ± SD. Data were analyzed either by two-tailed Student's *t*-test or by ANOVA for unpaired data from different experiments. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Myocardial infarct size

There was no significant difference in the AAR as a percentage of LV area among control I/R, vehicle, 6R-BH4, GA-BH_4, GA-6S-BH4, and L-NAME + GA-BH_4 groups. The infarct size as a percentage of the AAR was markedly reduced in the GA-BH4 group (22 ± 8%) compared with those in control I/R (66 ± 8%, $P = 0.0001$), vehicle (65 ± 10%, $P = 0.0001$) and L-NAME + GA-BH_4 (62 ± 10%, $P = 0.0001$) groups (n = 8/group) 16 h post-myocardial infarction (Fig. 1). In contrast, GA-6S-BH_4 treatment only slightly reduced myocardial infarct size compared to that in control with value of 52 ± 10% ($P = 0.01$, n = 8/group); and 6R-BH_4 did not reduce myocardial infarct size compared with that in control. 6S-BH_4 is the stereo-isomer of 6R-BH_4 and is 60-times less potent as NOS cofactor than 6R-BH4 [29]; and oral exogenous BH_4 has no effect on endothelial function owing to systemic and vascular oxidation of BH_4 [30]; therefore, these data indicated that GA-BH4 has definitely more cardioprotective effect *in vivo*. Moreover, pretreated with L-NAME *in vivo* to block NOS, GA-BH_4 supplementation failed to decrease myocardial infarct size, confirming the role of the NOS–NO pathway in the cardioprotective effect of BH_4.

3.2. Physiologic studies

According to echocardiography and cardiac catheterization 14 days post-myocardial I/R, the control I/R, vehicle-, 6R-BH_4-, and GA-6S-BH_4-treated rats showed decreased LV ejection fraction percentage, LV fractional shortening percentage, and ± dP/dt (n = 6/group, Fig. 2).

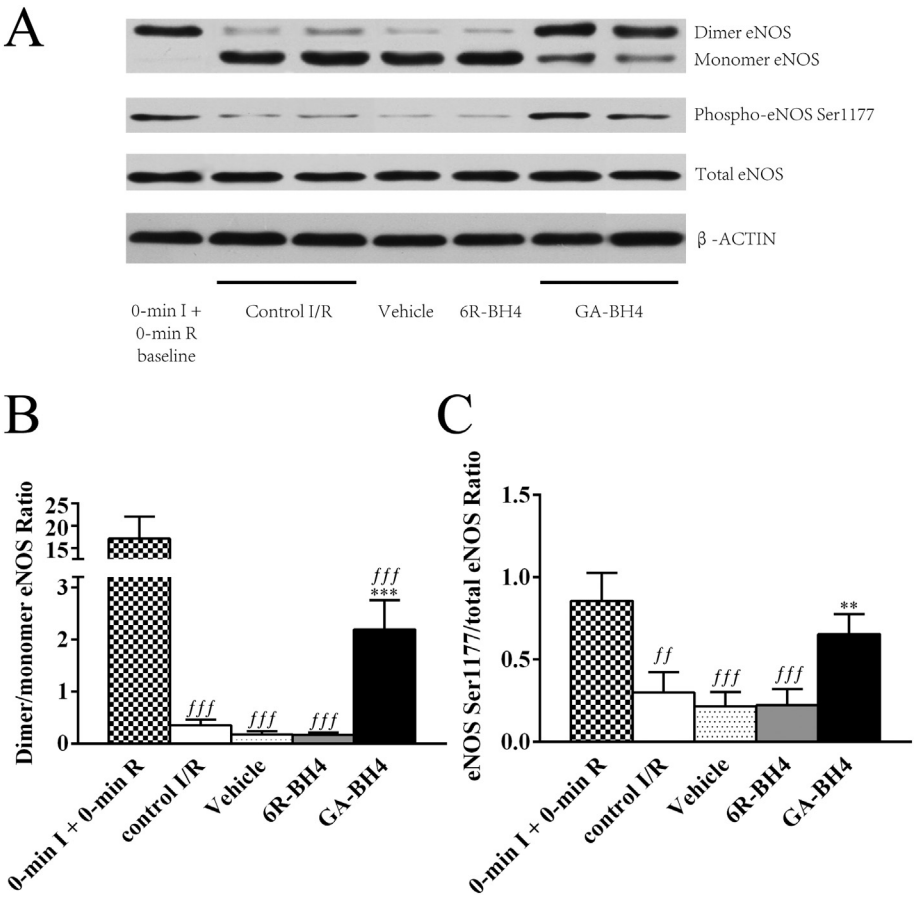

Fig. 6. Western blot analysis for eNOS in the cardiac homogenates of area at risk. A. Representative Western blots for dimers, monomers eNOS, total eNOS and eNOS Ser1177 phosphorylation from non-ischemia normal (0-min I + 0-min R baseline), control I/R, vehicle, 6R-BH_4 or GA-BH_4 groups at equal protein loading. B–C: graphic presentation of densitometric data. Values are means ± SD. N = 5/group. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control I/R group, $ffP < 0.01$, $fffP < 0.001$ *vs.* 0-min I + 0-min R (baseline).

GA-BH_4 treatment significantly improved each of these conditions, indicating the improvements of cardiac function in chronic phase of post-I/R. There was no difference in systolic and diastolic blood pressures and heart rate among the five groups before, during, and after 60 min of ischemia.

3.3. Fibrotic area

Fibrotic area as a percentage of the LV was significantly reduced in the GA-BH_4 group (24 ± 4%) compared with the control I/R (59 ± 7%, $P = 0.0001$), vehicle (51 ± 7%, $P = 0.0001$), 6R-BH_4 (54 ± 10%, $P = 0.0001$) and GA-6S-BH_4 (42 ± 7%, $P = 0.0001$) groups 14 days post-myocardial I/R (n = 6/group, Fig. 3).

3.4. BH4 levels

3.4.1. In myocardium during ischemia and reperfusion

BH_4 tissue levels were assessed by HPLC. Values are expressed as mean ± SD (n = 6/group) in nmol/g tissue. BH4 levels in the AAR in control I/R rats began to decline after 20-min ischemia and were greatly depleted to 0.037 ± 0.008 after 40-min regional ischemia compared to those in non-ischemia normal (NI) hearts (0.119 ± 0.030). The low levels of BH_4 in the AAR were continuously maintained by 60-min ischemia (Fig. 4A). Subsequently, early 5-min reperfusion following ischemia cause further BH4 depletion (0.012 ± 0.009). After 10 min of reperfusion, BH_4 levels in the AAR began to rise inversely. By 16 hours reperfusion, BH4 levels returned to half of basal values (0.057 ± 0.019) and this decrease was maintained throughout the entire observation/reperfusion period (48-h R: 0.061 ± 0.025; 14-d R: 0.062 ± 0.021). All of these changes of myocardial BH_4 levels in the vehicle group (Fig. 4C) were similar to those in the control I/R group. The sharp decline of BH4 levels in the ischemic area during ischemia and/or early reperfusion indicated that BH_4 depletion in myocardium could be concerned with myocardial I/R injury.

3.4.2. In myocardium with GA-BH4 administration

The effects oral GA-BH4 administration on myocardial BH4 levels before and following ischemia and/or reperfusion were determined. Myocardial BH4 levels began to rise 6 h after oral GA-BH_4 administration and increased to maximal concentration (> 0.7 nmol/g tissue) 8 h after GA-BH_4 administration. However, oral uncoated 6R-BH_4 administration had no effects on changes of myocardial BH4 levels (Fig. 4B). Significantly, oral GA-BH_4 administration was associated with a massive increase in myocardial biopterin concentrations following ischemia (20-min I: 0.212 ± 0.069, 40-min I: 0.210 ± 0.068, 60-min I: 0.161 ± 0.063), as well as after reperfusion (5-min R: 0.775 ± 0.169, 10-min R: 0.776 ± 0.164, 16-h R: 0.818 ± 0.185). Values were up to 3–62 folds higher compared with those in the AAR of the control I/R, vehicle and 6R-BH_4 groups (Fig. 4C). Then, BH_4 levels in the AAR displayed augmented levels 48 h post-myocardial I/R in comparison to the control I/R. This confirmed that the oral administered GA-BH_4 reached the heart and maintained myocardial BH4 levels during the I/R period.

3.5. eNOS activity in the AAR of rat heart

Myocardial eNOS enzyme activity in the AAR were measured by analyzing the conversion of [3H] L-Arginine into [3H] L-Citrulline (Fig. 5, n = 5/group).We found that myocardial eNOS activity was significantly decreased in the AAR of the control I/R group after 60 min-ischemia (60-min I/0-min R, decreased by 75%), 60-min ischemia and 5-min reperfusion (60-min I/5-min R, decreased by 83%) and 60-min ischemia and 10-min reperfusion (60-min I/10-min R,

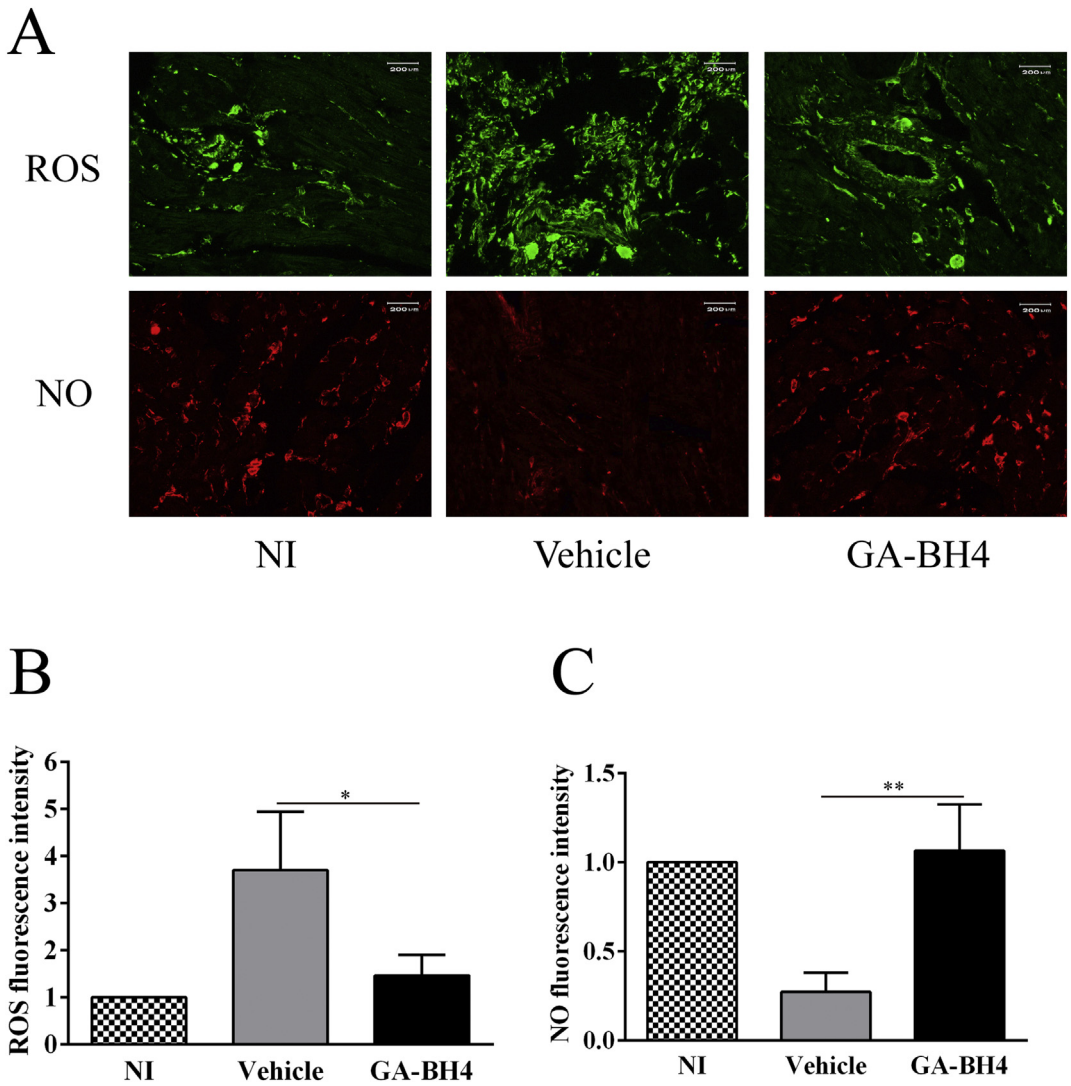

Fig. 7. *In situ* detection of reactive oxygen species (ROS) and nitrate oxide (NO). A, Representative fluorescent images of ROS (top) and NO (bottom) in left ventricular (LV) myocardium sections from non-ischemia normal (NI, left column), vehicle- (middle column) or GA-BH4 (right column) groups. Upper ROS and lower NO images show small blood vessel (s) and adjacent myocardium of LV (bar = 200 μm). B − C, Bar graphs for the total intensity of fluorescence. Generation of ROS (B) and NO (C) were evaluated by the fluorescent intensity of DCF and DAR-4M, respectively. Data are mean ± SD of at least 20 focal fields per animal from four rats in each group. All data of fluorescent intensity were normalized to that in NI hearts. $^{*}P < 0.05$ and $^{**}P < 0.01$.

decreased by 50%) compared to baseline (0-min I/0-min R). However, when the rats were subjected to oral GA-BH_4 administration 24 h before myocardial ischemia, significantly marked restoration of eNOS activities were detected during myocardial I/R (decreased by 17–21% compared to baseline), but I/R-induced reduction of eNOS activity was not attenuated by supplementation with vehicle or 6R-BH_4. These results suggested that oral GA-BH4 pretreatment could improve eNOS function and protect cardiac function against myocardial I/R injury.

3.6. Myocardial eNOS protein levels

Immunoblotting was performed for total, phosphorylated (Ser1177 phosphorylation), dimer and monomer eNOS in the AAR of rat hearts (Fig. 6, n = 5/group). Compared to baseline (0-min I/0-min R), the ratio of activated phosphorylated eNOS (p-eNOS)/total eNOS protein was clearly ~74% reduced after 60-min I/5-min R in the control I/R group. In the GA-BH4 group, the ratio of p-eNOS/total eNOS protein was higher with only a maximum 35% decrease compared to baseline after 60-min I/5-min R. However, the ratios of p-eNOS/total eNOS protein in the vehicle and 6R-BH_4 groups were both similar to that in control I/R group. Moreover, the myocardial eNOS protein dimer/monomer ratios were also decreased in the control I/R (0.36 ± 0.07), vehicle (0.18 ± 0.06) and 6R-BH_4 (0.17 ± 0.02) groups, only 1.2–2.5% of baseline (0-min I/0-min R, 14.20 ± 3.04) after 60-min I/5-min R. GA-BH_4 pretreatment significantly prevented the decrease of the ratio in the AAR after 60-min I/5-min R (4.43 ± 0.85), > 10 times the ratio of control I/R group. These findings thus suggested that GA-BH4 pretreatment is able to diminish the detrimental effects of I/R on myocardial eNOS, preserving its activation and dimerization.

3.7. Myocardial ROS and NO generation

Fig. 7 shows ROS and NO imaging in NI hearts and vehicle- and GA-BH_4-treated hearts after 60-min ischemia and 5-min reperfusion (n = 4/group). Generation of ROS and NO in myocardium were evaluated by the fluorescent intensity of DCF and DAR-4M, respectively. There was robust increase in DCFH fluorescence (green) in vehicle-treated hearts after I/R (3.71 ± 1.24 fold compared with NI hearts) and GA-BH4 administration significantly diminished ROS generation (1.47 ± 0.44fold compared with NI hearts, $P = 0.014$ *vs.* vehicle

group). Meanwhile, oral GA-BH4 administration clearly increased NO generation (red fluorescence) following I/R (1.07 ± 0.26 fold) compared to vehicle (0.27 ± 0.11 fold, $P = 0.0014$). These findings provide strong evidence that pre-ischemic oral GA-BH4 administration improves NOS function in myocardium during I/R with preserved NO, but decreased ROS generation, alleviating the I/R-induced myocardial injury.

4. Discussion

I/R induces oxidative stress and increases the intracellular levels of ROS, resulting in tissue damage [31]. ROS is generated by mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, xanthine oxidase and NOS [32,33]. As vascular endothelium and cardiac myocytes are exposed to increased oxidant stress, NOS is converted to a ROS generator from a NO source due to "NOS uncoupling" [34]. The most important reason of NOS uncoupling is believed to be the lacking of the critical cofactor BH_4 of NOS, and many reports have been published to preserve eNOS function and ameliorate heart dysfunctions by BH_4 supplementation [13,35,36]. Nevertheless, BH_4 is highly redox-sensitive and easy to be oxidized by one or two electron reactions to generate BH_3^+ radical and BH_2 [37] that lack eNOS cofactor activity. It is therefore suggested that oxidative stress leads to excessive oxidation and depletion of BH_4. Thus exogenous BH_4 supplementation is frequently poor effective *in vivo*. In order to increase its activity and stability, in this study we coated BH_4 with a novel encapsulation of Gum Arabic for potential application in various industries. Our results demonstrated that oral GA-coated 6R-BH4 supplementation prior to myocardial ischemia resulted in a significant enhancement in myocardial BH_4 levels, compared with inefficient uncoated 6R-BH4 treatment (Fig. 4B and C). Thus, the GA-encapsulation process helps to stabilize exogenous BH_4 by preventing it from being oxidized by oxidative stress during I/R.

eNOS proteins are all homodimers. A functional eNOS transfers electrons from NADPH, *via* flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in the carboxy-terminal reductase domain, to the heme in the amino-terminal oxygenase domain, where the substrate L-arginine is oxidized to L-citrulline and NO with the essential cofactor BH_4 binding [38]. As a consequence of BH_4 depletion due to oxidative stress, uncoupling eNOS is converted from NO formation into a dysfunctional generating $\cdot O_2^-$ enzyme. As shown in Figs. 5 and 6, we observed ~75% loss of eNOS activity after 60 min of ischemia and even more (83%) dysfunction during early 5 min of reperfusion; subsequently, it began to recover to 50% of basal levels after 10 min of reperfusion. These results demonstrated that eNOS activity well-correlated with the myocardial BH4 levels (Fig. 4A) where a significant loss of BH4 was seen after ischemia and early reperfusion. Contributing to the increase of BH_4 levels in myocardium with pre-ischemic oral GA-BH_4 supplementation, eNOS activity, activated phosphorylated eNOS levels and its dimerization were well preserved during myocardial I/R.

In the uncoupled state, electrons normally flowing from the reductase domain of eNOS to the oxygenase domain are diverted to molecular oxygen rather than to L-arginine; in these conditions, $\cdot O_2^-$ rather than NO is produced [34], which indicates eNOS dysfunction. In this study, we confirmed cardiac eNOS dysfunction and ROS generation by the fluorescent indicators DCFH and DAR, respectively [28]. Intracellular NO production level can be detected by the fluorescence probe of DAR, characterized by higher photostability, longer excitation wavelength and a wide pH range. Hence, DAR compounds are useful for bioimaging NO in samples [39]. Moreover, nonfluorescent DCFH can be converted to green fluorescent DCF upon intracellular oxidation as a qualitative marker of cellular oxidative stress to estimate the formation of ROS, such as $\cdot O2^-$, H_2O_2 and $ONOO^-$ [40]. Therefore, the use of DAR plus DCFH in our technique facilitated simultaneous detection of NO and ROS production. As shown in Fig. 7, strong DCF immunofluorescent staining for ROS was shown in hearts of I/R rats but not in NI hearts, while much weaker DAR-4M fluorescence displaying NO production was detected in I/R hearts compared with healthy ones. Oral administration of GA-BH_4 ameliorated eNOS function, as determined by increased cardiac NO production and decreased cardiac ROS generation. The findings also demonstrated the existence of uncoupled eNOS in the endothelium and myocytes.

Importantly, we observed significantly the reduced myocardial infarct size, the improved left ventricular function and the inhibited fibrosis after I/R with oral GA-BH_4 supplementation before ischemia (Figs. 1–3). Usually, there is a view that the antioxidation induced by reducing agents might be one of the most likely mechanisms contributing to the protective effect against I/R injury. Considering the antioxidant effect of GA [41] and 6S-BH_4 [29] on scavenging of ROS generated, the vehicle (GA) and GA-6S-BH_4 treatments were performed simultaneously in this study. As the results shown, GA, as well as GA-6S-BH_4, administration has no or slight effects against myocardial I/R injury. Therefore, it is concluded that the protective effects on acute and chronic myocardial I/R injury might be associated with preserved eNOS enzyme function secondary to significantly higher myocardial BH_4 levels. Thus, the results demonstrated that BH4 depletion is a critical factor in acute and chronic I/R injury and that its oral supplementation with GA-coated can confer protection.

In summary, our data indicate that the encapsulation of BH_4 in GA results in better exogenous BH_4 stability. Oral administration of GA-BH_4 provides protection against myocardial I/R injury, possibly by maintaining myocardial BH_4 levels, preserving eNOS function and inhibiting ROS generation. The increased stability and the high activity of the encapsulated BH_4 could make this approach an attractive choice for clinical applications.

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Conflicts of interest

The authors declare no conflict of interest.

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