Improvement of cardiac dysfunction by L-arginine combined with vitamin C through restoring angiogenesis associated with NADPH/NOS/NO signal pathway in diabetic rats

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Abstract

Background

In diabetic patients, the occurrence of ischemic events such as myocardial infarction is significantly higher than that of normal patients. L-arginine and vitamin C are commonly used adjuvant drugs in clinical practice. But whether L-arginine and vitamin C can be used together, the role of promoting angiogenesis to improve vascular dysfunction and its mechanism of action are not clear. To this end, we investigated whether L-arginine in combination with vitamin C promotes revascularization after myocardial infarction in patients with type 2 diabetes by inhibiting angiogenesis disorders associated with NADPH oxidase.

Methods

Experimental myocardial infarction (MI) was induced by left anterior descending coronary artery ligation in diabetic Sprague-Dawley rats and non-diabetic controls. Cardiac function was studied by echocardiography and LDH activity. Western blotting was used to study VEGF, Nox4, Nox1, Nox2, eNOS and phospho-eNOS (Ser1177), CAT-1. Angiogenesis, cell proliferation and migration were detected by micro-vessel density assay, cell viability assay, scratch wound-healing assay and tube formation assay.

Results

First, we cultured rat aortic endothelial cells in a high glucose environment for 48 hours, then treated L-arginine and vitamin C alone or together. It was shown that high glucose significantly damaged cell proliferation, migration and tube formation, which was normalized by L-arginine with vitamin C combined application. Furthermore, we found that co-treatment of L-arginine and vitamin C activated NOS/NO signaling pathway via inhibited NADPH oxidase activity, which regulated angiogenesis in RAECs. In vivo, L-arginine and vitamin C co-intervention improved cardiac function by implementing echocardiographic examination and LDH activity. This therapy also relieved the vasodilatory response to acetylcholine and restored the expressions of p-eNOS and VEGF, together with reducing Nox2 and iNOS production in the ischemic myocardium of high-fat diet (HFD)-fed rats following streptozocin (STZ) injection.

Conclusion

Overall, for the first time, this study reveals that the combination of L-arginine and vitamin C restores the blood supply to myocardial infarction in rats with type 2 diabetes by promoting angiogenesis, which was attributed to activating NOS/NO signaling pathway via blocking NADPH-dependent O_2^- generation.

Background

Epidemiological studies revealed that myocardial ischemia/infarction is the leading cause of morbidity and mortality in the patients with diabetes mellitus (DM) [16, 41]. A population-based study also showed that the incidence of myocardial infarction (MI) in diabetic patients is significantly higher than non-diabetic patients[12, 13]. DM leads to the impairment of myocardial angiogenesis[3, 4], which forms new vessels to provide oxygen and nutrient supply to the ischemic area of MI. Thus, the improvement of angiogenesis is considered as an innovative therapeutic approach for the treatment of ischemic heart disease[1, 2].

Nitric oxide (NO), which is synthesized from L-arginine via endothelial nitric oxide synthase (NOS), plays an important role in the regulation of angiogenesis. NO is demonstrated to inhibit apoptosis by decreasing caspase-3, and enhance endothelial cell migration and proliferation in part by increasing the expression of VEGF[4]. The release of endothelium-derived NO is reduced in ischemic heart disease patients[32]. In fact, angiogenesis is attenuated when NO bioactivity is reduced[4].

L-Arginine, a so-called “conditionally” essential amino acid, is a substrate in the synthesis of NO by the enzyme NOS[4, 5]. Some studies found that L-arginine enhanced angiogenesis, for example, L-arginine enhanced angiogenesis in the hypoxic pulmonary circulation, which may attenuate hypoxic PH by producing new parallel vascular pathways through the lung[18]. However, most studies regarded L-arginine as one of combination therapies in angiogenesis. For example, L-arginine administration presumably caused additional effects on exercise-induced angiogenesis by promoting VEGF expression in the left ventricle in middle-aged rats[43]. Placental growth factor (PlGF) and L-arginine have a more pronounced and synergistic protective effect on myocardial protection in a rat model of acute myocardial infarction[25]. L-arginine supplementation to FGF-2 therapy had myocardial microvascular reactivity and perfusion in a porcine model of endothelial dysfunction[46].

Vitamin C is the main water soluble antioxidant in human plasma[10] with potential therapeutic benefit in protecting against endothelial dysfunction in several clinical situations[14]. Vitamin C may have beneficial effects on nitric oxide bioavailability induced by L-arginine[44]. The beneficial effects of L-arginine and vitamin C co-supplementation have been recently documented in some studies. For example, L-arginine dependent coronary segment vasodilatation augmented by the antioxidant vitamin C in patients with coronary artery disease. However, whether administration of combined administration of vitamin C and L-arginine can lead to additional improvement of cardiac function and pathological amelioration in diabetic rats with MI has not been investigated. In present study, we therefore examined the synergistic effects of L-arginine and vitamin C on the MI through promoting angiogenesis in diabetic rats.

Materials And Methods

Induction of T2DM model

Male Sprague-Dawley (SD) rats weighing 130–150 g were provided by Qinglongshan Lab Animal Ltd, Nanjing, China. All animals were treated strictly in accordance with the Guidelines of Animal Experiment set by the Bureau of Sciences and Techniques of Jiangsu Province, China [NO. SYXK2007–0025]. The experimental protocols were approved the ethic committee of China Pharmaceutical University.

Rat model of T2DM was performed as previously described[23]. SD Rats were fed with a high-fat diet (HFD, 22 g/d) consisting of 10% saccharose, 10% lard, 10% sugar, 5% egg yolk powder, 0.5% cholesterol, 74.5% basal chow. The ordinary chow was constituted by 36% corn, 23% triturate wheat, 10% bran, 12% soy bean powder, 3% egg; 12% fsh powder, 2% driedyeast, 1% of amixture of calcium bicarbonate, multi-vitamins and micro-elements. Both the normal chow and HFD were purchased from Qinglongshan Lab Animal Ltd (Nanjing, China). After 1 week of low-dose STZ injection (40 mg/kg, dissolved in pH 4.5 citrate buffer), HFD-fed rats were recognized as diabetic while the fasting blood glucose (FBG) level reached 16.7 mM.

SD rats were randomly allocated to six groups: Control sham group received only chest open without ligation of left anterior descending artery (LAD); T2DM sham group received only chest open without LAD; T2DM + MI group performed by LAD, which was treated with or without L-arginine (500 mg/kg, dissolved in 2 mL water, i.g.), or Vitamin C (50 mg/kg, dissolved in 2 mL water, i.g.) or both. All the intervention group was conducted once a day for 2 weeks after MI surgery. The untreated rats were gavage fed daily with 2 mL water to exclude effects of the feeding procedure.

Induction of myocardial infarction in rats

After establishing of T2DM model, myocardial infarction were performed as described in previous studies[11, 31, 36]. Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and additional injection of pentobarbital was prepared for requirement. Then rats received tracheal intubation and artificially ventilated (70 strokes/min, 8–10 mL/kg tidal volume) and the vital signs were continuously monitored by electrocardio-graphic monitoring during the experiment. After the fifth intercostal space thoracotomy, the pericardium was removed and left anterior descending artery was permanently ligated with a silk thread 6 − 0 approximately 1 to 2 mm below the left auricle. Once the ligation succeeded, left ventricular myocytes would turn pale and a huge R wave appearing with a notch in electrocardiography monitoring, which was considered as a criterion for achieving a proper model of MI. The chest was then closed with air exhaust in order to restoring intrathoracic negative pressure. At the end of surgery, penicillin sodium (800U/kg, i.m.) were given once a day for 3 days in case of wound infection. During the first 12 hours, rats were permitted freely access to water, while food were provided 12 hours later.

Serum biochemical measurement

Blood samples from rats were collected and centrifuged at 3000 g for 10 min at 4 °C. The serum was collected and stored at -80 °C before use. FBG and fasting insulin (FIN) were assayed following instructions of the kits provided by Halin Biological technology co., LTD (shanghai China). The level of nonesterified fatty acid (NEFA), total cholesterol (TC), triacylglycerol (TG), low density lipoprotein

cholesterin (LDL-C), high density lipoprotein cholesterin (HDL-C) and lactate dehydrogenase (LDH) were detected by rat assay kit provided by the Nanjing Jiancheng Bio engineering Institute (China).

Measurement of myocardial infarct area

Rats were anesthetized and hearts were then removed and frozen at -80 °C for 30 min. The frozen hearts were sliced into 5 slices with approximately 2 mm thickness. The sections were incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC, BU Technology CO, LTD) at 37 °C for 15 min in the dark environment. The slices were flipped once every 5 min for the even dyeing. After thorough washing, the slices were fixed with 10% formalin overnight and then photographed by a digital camera. The infarct areas were calculated using ImageJ software.

HE staining analysis

The HE staining was performed as described previously[48]. Briefly, hearts were removed from the rats and fixed with 4% paraformaldehyde overnight, and then embedded in paraffin. The prepared sections were sliced to 8 μm thick and stained with hematoxylin and eosin (HE). Morphometric evaluation of the tissue was performed by a light microscopy.

Cardiac function assessment

In order to assess the myocardial function, rats underwent echocardiographic examination of the heart under anesthesia via a Vevo 2100 (Visual Sonics, Toronto, Canada). Representing left atrial diameter, two-dimensional (2D) mode images of parasternal short-axis plane were recorded. Moreover, motion mode (M-mode) tracing was taken below the tips of the mitral valve leaflets. For evaluating left ventricular structural changes, several parameters from M-mode were measured. Left ventricular end diastolic volume (LVEDV), left ventricular end systolic volume (LVESV), along with LV mass were measured as indexes of cardiac diastolic function. And with the aid of some M-mode detection, left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD),left ventricular fraction shortening (LVFS) and left ventricular ejection fraction (LVEF) were calculated[9].

$$LVEDV = (7 \times LVEDD3)/(2.4 + LVEDD)$$

$$LVESV = (7 \times LVESD3)/(2.4 + LVESD)$$

$$LVFS\ (\%) = ((LVEDD - LVESD)/LVEDD) \times 100.$$

$$LVEF\ (\%) = ((LVEDV - LVESV)/LVEDV) \times 100.$$

NO level and NOS activity assay

The kits of NO and NOS were used to determine the level of serum NO and NOS activity (Nanjing Jiancheng Bio engineering Institute, China) as we applied before[23]. For the detection of NO production, samples, standards and HRP-labeled antibodies were added into the glutathion peroxidase (GSH-PX) coated micropores. After incubation and washing, the plasma NOx (nitrite and nitrate) level using nitrate

reductase method was detected with the help of the substrate TMB coloration. TMB was catalyzed by peroxidase turning to blue, and converted to final yellow under the action of acid. Color was positively correlated with GSH-PX. The absorbance (OD value) was measured at 450 nm and the sample concentration was calculated.

The quantification of NOS activity was measured according to previous study[33]. NOS catalyzes the reaction of L-arginine with molecular oxygen to generate NO, bringing colored compounds with nucleophilic substances. The total NOS activity was calculated at 530 nm. Depending on the nature of more sensibility to Ca2 + of eNOS than iNOS, the activity of eNOS was monitored when pretreated with Ca2 + chelator. Therefore, iNOS activity can be calculated as the difference value between total NOS activity with eNOS activity.

Immunohistochemical analysis

Immunohistochemistry in the heart tissues was employed to investigate the expression of VEGF, Nox2, eNOS and iNOS. Heart tissues were extracted following embedded in OCT compound (Tissue-Tek) and snap-frozen in liquid nitrogen. After sectioned as slices of 8 μm thickness, samples were blocked by PBS (1% BSA and 0.3% triton X-100) for 45 min. Then slices were overlaid respectively by anti-VEGF (1:200, CST), anti-Nox4 (1:200, Abways), anti-eNOS (1:150, Proteintech) and anti-iNOS (1:150, Proteintech) at 4°C overnight. Afterwards, slices were incubated with the rabbit anti-goat biotinylated secondary antibody (dilution 1:100, Abways) for 2 hours at room temperature. Then use diaminobenzidine (DAB) chromogen kit (Beyotime Biotechnology co, LTD.) to stain as the chromogen. Sections were counterstained with hematoxylin before examination under OLYMPUS CX31 microscope.

Micro-vessel density (MVD) assay

MCD was measured by CD31 immunohistochemistry (an angiogenesis special marker) to assess angiogenesis in the myocardium. The heart slices were incubated with anti-CD31 (1:250, Santa Cruz) followed by dylight 594-conjugated anti-rat immunoglobulin G (1:500, Abways) for 1.5 h. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). MVD was calculated by the number of CD31 + objects per field and counting at low power lens[22]. Three random heart sections were analyzed per rat.

Primary cell culture

Rat aortic endothelial cells (RAECs) were isolated from normal SD rats[20]. RAECs were cultured in 5 ml low-glucose DMEM (Hyclone, USA) medium containing 10% FBS (WISENT), 1% antibiotics (Hyclone, USA) and endothelial growth factor (EGF, 10ug/mg). After identified by von willebrand factor (vWF)[24], an endothelial cell marker, primary RAECs of 4 to 6 generations were used in the experiments.

Western blot analysis

Western blot analyses were performed as previously reported[23]. RAECs were scraped in radio immunoprecipitation assay (RIPA) lysis buffer (BU Technology CO, LTD) for 15 min at 4 °C. After centrifuged at 4 °C, 14 000 g for 15 minutes, the supernatant containing cell protein was collected and

determined by BCA protein quantitative kit (BU Technology CO, LTD). Cell protein was separated by SDS-PAGE and transferred to PVDF membranes (0.45 μm, Millipore Co. Ltd.). Membranes were blocked with 5% non-fat milk (5% w/v) in Tris-buffered saline with Tween-20 for 1.5 h and incubated at 4℃ with primary antibodies of anti-VEGF (1:1000, CST), anti-Nox4 (1:1000, Abways), anti-Nox1 (1:1000, Abways), anti-Nox2 (1:1000, Abways), anti-eNOS (1:1000, Proteintech), anti-iNOS (1:1000, Proteintech), anti-phospho-eNOS (Ser1177, 1:1000, Santa Cruz), anti-CAT-1 (1:1000, CST) in 1% non-fat milk in PBST overnight. After then, the membranes were incubated with the goat anti-Rabbit IgG (1:5000, Abways) as secondary antibody at room temperature for 1.5 h. The blots were developed using an ECL Kit (BU Technology CO, LTD) and the densitometric analysis of blots was performed with ImageJ software (NIH, Littleton, CO, USA).

Cell viability assay

Cell viability was examined by performing 3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2-H- tetrazolium bromide (MTT, 5 mg/mL, Solarbio) assay. RAECs were prepared in 96-well plates and incubated with corresponding treatment. Arriving at time point, cells were added MTT (0.5 mg/mL) at 37℃ for 4 h in darkness. Afterwards, the medium was replaced with 200 μL dimethyl sulfoxide to solve the precipitated formazan. The absorbance of each well was measured at 490 nm by a microplate reader (POLARstar Omega®, BMG, Germany).

Scratch wound-healing assay

The migration of RAECs was determined by scratch wound-healing assay. RAECs were seeded in 6-well plates. When developing as a confluent monolayer, cells were scratched straightly with 200 μL sterile pipette tips to generate a cell-free zone. Cell fragments around the scratches were washed using PBS. After 24 h culture, the images were taken under a microscope. The images of the wounded area were photographed immediately (time 0) and 24 hours after scratch. The wound areas were calculated by image J software (NIH, Littleton, CO, USA). Wound closure was quantified as percentage of initial wound area that had been recovered by VSMCs,% Wound Closure = (areat0-areat24)/areat0 × 100%.

Tube formation assay

The tube formation assay is a widely-applied tool to detect cell angiogenesis[6, 47]. Matrigel (BD Biosciences, USA) was added in the 96-well plates and allowed to polymerize for 30 min at 37℃. RAECs were seeded in the coated plate at appropriate density and incubated with normal medium until cell adherence. After treatment, the tube formation of RAECs was observed and randomly photographed using a microscope. Number of tubules and branch points of each group were calculated with ImageJ software.

Measurement of O_2^- production

The oxidative fluorescent dye dihydroethidium (DHE, Sigma) was used to measure total O2- production. DHE was oxidized by intracellular O2- and then incorporated into the chromosomal DNA in the nucleus to generate red fluorescence. After culturing in 6-well plate, RAECs were treated and then washed twice using

PBS. When prepared as 10 μM solution diluted in serum free medium, 2 mL DHE solution was put in each well for 30 min in the darkness. After final washing, the fluorescence images were observed by fluorescent microscopy (Olympus IX53, Olympus, Tokyo, Japan). The fluorescence intensity was measured using ImageJ software and the cellular O2- level was indicated as the ratio of fluorescent intensity to that at basal level.

Measurement of $ONOO^-$ levels

The formation of peroxynitrite (ONOO-) was assessed in RAECs by an ELISA kit (RENJIEBIO, shanghai) according to manufacturer's instructions. After adding 100 μl H2SO4 to stop the reaction, the prepared 96-well plate was detected by a microplate reader at absorbance of 450 nm. The ONOO- levels were calculated by the difference of OD value between samples and negative control.

Statistical analysis

Data are presented as the mean ± SE. Significant differences between and within multiple groups were examined using analysis of variance for repeated measures, followed by Duncan's multiple-range test. Student's t test was used to detect significant differences between two groups. $P < 0.05$ was considered statistically significant.

Results

L-arginine and vitamin C ameliorates cardiac function

Following an injection of STZ in rats with HFD, blood fasting blood glucose (FBG) and homeostasis model assessment of insulin resistance (HOMA-IR) was significantly elevated relative to control group (Fig. 4a, b). Simultaneously, the level of NEFA, TC, TG and LDL-C significantly increased in T2DM rats (Fig. 4c, d, f, g), compared to control rats, with HDL-C conversely decreased (Fig. 4e). Thus, a successful T2DM model was established in vivo study. The intervention with L-arginine and vitamin C in the last 2 weeks resulted in an obvious reversion in these biochemical indexes, implying that L-arginine single use and combination use with vitamin C could attenuate metabolic disorders and systemic insulin resistance in T2DM rats. What's more, adjunctive therapy played a more significant role.

Given that combination of L-arginine and vitamin C mediated angiogenesis after AMI injury in T2DM rats, we next examined the role of combination medication on cardiac function, which was analyzed by echocardiography. At 2 weeks after MI, heart systolic function was considerably impaired, as indicated by the LVEF and LVFS (Fig. 3f, i). Transthoracic echocardiography demonstrated significantly improved LVFS and LVEF in the combination group compared with the AMI injury group in diabetic rats. Furthermore, the increase in LVEDD (Fig. 3g) and LVESD (Fig. 3j) is a typical index for cardiac dilation. Representative M-mode images of 2D parasternal long axis by echocardiography (Fig. 3c) showed improvement in LV mass (Fig. 3e), LVEDV (Fig. 3h) and LVESV (Fig. 3k) in AMI of diabetic rats, but was significantly smaller in co-treated group. These results suggested that intravenous administration of L-arginine and vitamin C restored cardiac function after AMI injury in T2DM rats. In addition, as a tool of myocardia disease diagnoses, the activity of LDH in different group was further tested. As shown in

Fig. 3l, LV ligation directly gave rise to the increase of LDH, suggesting acute myocardial damage, and the abnormal increase of LDH was alleviated by L-arginine and vitamin C treatment.

L-arginine and vitamin C ameliorated angiogenesis in diabetic rats

Microvascular density assay is a cost-effective, simple and rapid method to evaluate angiogenic potential of medicine, which is widely used to determine angiogenesis in vivo. The immunostaining targeting CD31 was performed for the assessment of angiogenesis in the transversal section of the ventricles (Fig. 5a). As shown in Fig. 5d, the quantity of CD31-positive EC intensity dramatically decreased in the diabetic group and diabetic AMI group compared to control rats. After 2 weeks of L-arginine or vitamin C alone treatment, the number of CD31-positive cells and formation of new blood vessel.

Vascular endothelial growth factor (VEGF), a key angiogenic growth factor, stimulates proliferation, migration and tube formation of endothelial cells primarily through the VEGF receptor type2. VEGF is an important growth factor that promotes angiogenesis. As a major pro-angiogenic isoform, the protein level of VEGF can reflect the degree of angiogenesis. Moreover, NO is demonstrated to enhance endothelial cell migration and proliferation in part by increasing the expression of VEGF. On this basis, NO-VEGF pathway was analyzed to confirm the effects of L-arginine or vitamin C on angiogenesis by immunofluorescent analysis. A significantly decreased fluorescence of VEGF was observed (Fig. 5c) in the myocardium of diabetic rats and the definite increases of fluorescence could be noted in L-arginine or vitamin C-treated rats. The combined therapy with L-arginine and vitamin C was more remarkable in increasing CD31 immunostaining and VEGF immunofluorescence than the monotherapy.

NOS involves the regulation of downstream angiogenesis of combination treatment

Our research had proved that NO plays a critical role of VEGF-induced angiogenic vessels, which is in line with previous studies. NO synthase (NOS) has been known as essential to NO bioactivity. So, we evaluated the relative contribution of eNOS and iNOS isoforms to the proangiogenic effect of VEGF and the role of L-arginine and vitamin C on them. We first examined the expression of total eNOS, phospho-eNOS at Ser1177 (p-eNOS) and iNOS in RAECs of different treatment by western blot.

As shown in Fig. 6e, high glucose impaired the activation of eNOS, but after administration of L-arginine and vitamin C, p-eNOS/eNOS augmented compared to high glucose group. Conversely, in Fig. 6g, iNOS protein expression enhanced under the high glucose induction, which was reversed by the treatment of L-arginine and vitamin C. Notably, the effect of adjunctive treatment was clearly improved compared with single drug. To further explore the influence of NOS activity on NO bioavailability, we detected the NO production in RAECs. Though high glucose improved the expression of iNOS, the total production of NO diminished according to Fig. 6a-d. NOS-mediated NO production elevated due to L-arginine alone and

combined with vitamin C. However, single treatment of vitamin C had no significant effect on NO generation.

Simultaneously, we tested p-eNOS and iNOS expression on heart tissues in Fig. 5b. The result demonstrated that the combination treatment of L-arginine and vitamin C restored p-eNOS and iNOS expression in the myocardium in diabetic rats, which was consistent with the activity of eNOS and iNOS in the serum in diabetic rats. Meanwhile, the impairment of serum NO bioavailability contraction was markedly ameliorated in diabetic-injury rats after the co-administration of L-arginine and vitamin C, which was superior to the monotherapy (Fig. 5k). Taken together, these data demonstrated that the combination application of L-arginine and vitamin C significantly enhanced angiogenesis which was associated with the NOS/NO/VEGF pathway.

L-arginine and vitamin C enhanced angiogenesis in RAECs

The proliferation and migration rate of ECs from adjacent healthy endothelium is the key to successful angiogenesis. It has been proved that hyperglycemia is critical for EC angiogenesis hypofunction, thus 30 mM glucose was used to impair angiogenesis in vitro.

In the vascularization process, the proliferation and migration of ECs is crucial factors of angiogenesis and network formation[28]. The scratch assay was applied on measure migration in RAECs. RAECs were incubated with 30 mM glucose for 48 hours, and it was found that high glucose decreased migration compared to normal RAECs. Notable, as shown in Fig. 8b, e, the L-arginine and vitamin C co-intervention exhibited more effective recovery of wound area than L-arginine or vitamin C alone.

To assess cell viability in presence of L-arginine and vitamin C, MTT colorimetric assay was carried out. The results in Fig. 8a manifested that EC proliferation rate was significantly higher after 12 h incubation of L-arginine and vitamin C together, illustrating the proliferation-promoting role of conjunctive therapy. Meanwhile, cells with high glucose showed no significant changes compared to control group, illustrating the non-cytotoxic effect of 30 mM glucose concentration. The initial process of angiogenesis is the organization of individual endothelial cells into a 3-dimensional tube-like structure[51]. RAECs were seeded on the basement membrane matrix. As shown in Fig. 8c, f, g, L-arginine alone, vitamin C alone, and two drugs co-administration were treated on the high glucose induced RAECs, and co-administration group most significantly promoted the tube formation of RAECs. on the formation of capillary-like tube structures was impaired by HG treatment. Thus, co-treatment of L-arginine and vitamin C exerts obvious pro-angiogenic effect via promoting the proliferation, migration and tube formation of RAECs. In the western blot assay, the expression of VEGF decreased compared with control after high glucose induced. On the contrary, VEGF protein amount increased in response to L-arginine and vitamin C (Fig. 8d, h).

Co-administration of L-arginine and vitamin C modulates NOS activity via NADPH-induced oxidative stress

High concentrations of ROS cause apoptosis and cell death and oxidative stress is associated with the cardiovascular diseases including hypertension, heart failure, atherosclerosis and diabetes. Oxidative

stress is regarded as the cause of O2- elimination and reactive oxygen species (ROS) and reactive nitrogen species (RNS) activation, which develops from hyperglycemia and in turn, disturbs the process of angiogenesis and exacerbates cardiac dysfunction[49]. Taken the anti-oxidative prosperity of L-arginine and vitamin C into consideration, the fluorescent spectrometry of a fluorescent probe dihydroethidium (DHE) was applied to detect intracellular O2- production. Figure 7a-b showed that red fluorescence intensity in RAECs treated with high glucose was strong owing to produced O2- coupled with DHE probe, which was markedly attenuated by L-arginine and vitamin C co-intervention.

To dive deeper into the molecular mechanisms underlying the role of L-arginine and vitamin C in O2- production, we next focused on the NADPH oxidase activity, the activation of which produces O2- accumulation directly. To this end, we examined protein level of Nox1, Nox2 and Nox4, three main NADPH oxidases on ECs by western blot analysis (Fig. 7c-f). The western results showed an increase of these three protein expression in high glucose-induced RAECs, which reversed by L-arginine and vitamin C co-treatment. In vivo, the representative Nox4 immunohistochemical images verified it that when T2DM and AMI happens, NADPH oxidase output was pathologically enhanced. This tendency was restrained under the action of L-arginine and vitamin C co-application. In vitro experiment, we further evaluated the effect of apocynin (APO, a general inhibition of NADPH oxidase) on O2- production. As shown in Fig. 7a, NADPH oxidase blockade with APO blocked the excessive production of O2-, to an extent similar to that observed in drug-treated group. Then, when administrated with APO, eNOS activation was improved, with decreased iNOS activity. Similar changes were also observed in L-arginine with vitamin C. Therefore, inhibition of NADPH oxidase plays a critical role in mediating NOS activity of L-arginine and vitamin C joint use.

Combination therapy increases L-arginine uptake and decreases ONOO- generation to mediate angiogenesis.

Considering the beneficial properties of L-arginine with vitamin C on NADPH-derived oxidative stress inhibition, we investigated the underlying mechanisms of combination of them deeply. Under cardiac oxidative stress state, overproduction of O2- reacts with NO, forming a highly reactive intermediate, peroxynitrite (ONOO-). To determine whether ONOO- was lessened by L-arginine with vitamin C co-administration, ONOO- level in RAECs was monitored. As shown in Fig. 7i, high glucose injury raised the level of ONOO- leakages. Nevertheless, this tendency was revised by post-incubation of L-arginine and vitamin C. what worth noting is, RAECs treated with vitamin C or (L-arginine + vitamin C) displayed significantly greater ONOO- control than L-arginine single incubation. It was also observed that L-arginine with vitamin C had a slight better influence on ONOO- production than did vitamin C alone. These phenomena demonstrated that one benefit of L-arginine and vitamin C co-treatment is, exogenous L-arginine promoted NO biosynthesis, while vitamin C working as a potent antioxidant quenched existent O2- to reduce inactivation of NO.

As the enzymatic production of NO is dependent on the intracellular levels of arginine[40], L-arginine transport on the cell membrane is no doubt crucial for NO generation, ultimately for angiogenesis. To affirm the role of L-arginine with vitamin C combined application on arginine uptake, we detected cationic amino acid transporter-1 (CAT-1) expression in RAECs, which is the predominant candidates for eNOS governing and arginine transport. The respective bands in Fig. 7g-h showed that L-arginine with vitamin C significantly augmented CAT-1 abundance compared to reduced CAT-1 due to high glucose incubation. Also notable is the elevated amount of CAT-1 occurred in vitamin C group along with (L-arginine + vitamin C) group. Instead, in L-arginine group, there was no significant changes with a mild augment compared with high glucose-treated RAECs. Since oxidative stress impairs CAT-1 activity[37, 50], it was inferred that vitamin C plays an antioxidative role on arginine transport. In support of this notion, another advantage of L-arginine with vitamin C joint utilization is, after offering extracellular L-arginine by L-arginine treatment, vitamin C enhanced the amount of intracellular L-arginine by suppressing oxidative injury on arginine transporter.

Combined therapy improved damaged endothelial function in diabetic AMI rats.

This tightly regulated process involves the degradation of extracellular matrix, disruption of cell–cell contacts, migration and proliferation, and capillary tube formation of endothelial cells (ECs). We then assessed the contribution of eNOS and iNOS to angiogenesis, respectively. We blocked NOS signaling by using NG-Nitro-L-arginine Methyl Ester (L-NMMA, a non-selective inhibitor of NO), NG-L-monomethyl (L-NAME, a specific inhibitor of eNOS) and S-Methylisothiourea Sulfate (SMT, a specific inhibitor of iNOS), to test different functions of NOS on downstream angiogenesis. After confirmed the inhibition role of L-NMMA, L-NAME and SMT (Fig. 8b-h), EC proliferation, migration and angiogenesis were measured. The results demonstrated that the inhibition of eNOS by L-NAME abolished the enhanced activation of EC viability, wound healing and tube formation in L-arginine and vitamin C treated RAECs. Result of L-NMMA was the same as that of L-NAME. Rather, treatment with SMT restored angiogenic injury caused by high glucose to some extent. Taken together, it was inferred that co-treatment of L-arginine and vitamin C ameliorated high glucose-induced angiogenesis deficiency largely through facilitating eNOS-dependent NO production and blocking iNOS-dependent NO production.

Discussion

Major findings from the present study demonstrate that, NADPH/NOS-dependent NO generation was crucial for damaged angiogenesis of RAECs triggered by high glucose. Since L-arginine known as a NO donor, vitamin C acting as an antioxidant, we developed a new strategy in which L-arginine combined with vitamin C accelerates the deficiency of angiogenesis via mediating NADPH/NOS/NO/VEGF signaling pathway. Simultaneously, we elucidated that L-arginine and vitamin C co-administration elicited proangiogenic role after AMI injury under T2DM condition.

Angiogenesis, known as new blood vessel formation from existing vessels, supplies impaired tissue adequate blood flow and oxygenation. The higher rate of myocardial angiogenesis is a compensatory mechanism secondary to the ischemia developed by coronary artery disease and other myocadiac diseases. It has been increasingly noticed that therapeutic angiogenesis is an attractive tool for diabetic cardiac ischemia by improving blood flow and providing oxygen to form new coronary collateral vessels. Previous studies revealed that diabetics exhibit a diminished angiogenic response to myocardial ischemia likely as a result of hyperglycemic environment[42]. The process of neovascularization deficiency of myocardial infarction caused by hyperglycemia is demonstrated by Fig. 1. Previous evidence has revealed that the single administration of L-arginine[25] or Vitamin C[27] exquisitely enhances vascularity in ischemic hearts, acting as a protective role on cardiovascular diseases, respectively. Thus, we hypothesized that the combination therapy of L-arginine and vitamin C is a promising therapeutic option that plays a better myocardial protection effect than single use hyperglycemia-impaired angiogenic response in diabetic AMI. The proliferation and migration of endothelial cells (ECs) is the initial process of coronary collateral vessel formation, thus we extracted primary rat aorta endothelial cells (RAECs) to implement in vitro experiments. Long been regarded as the root cause of on cardiovascular complications under diabetes mellitus (DM), exposure to hyperglycemia leads to angiogenic inadequacy and other endothelial dysfunctions in DM. First, we addressed whether high glucose stimulation distributes angiogenesis in RAECs in vitro and the role of L-arginine and vitamin C co-treatment on it after high glucose incubation. Following high glucose stimulation for 48 h, we treated RAECs with media containing combination of L-arginine with vitamin C or single drug for 12 h. By detecting cell viability assay to determine proliferation, wound scratch assay to determine EC migration and in vitro sprouting assay to determine tube formation ability, it was demonstrated that conjunctive treatment of L-arginine with vitamin C promoted EC proliferation and migration, accelerated insufficient angiogenesis disrupted by high glucose incubation. As the most pivotal angiogenic factor, VEGF is indispensable for driving endothelial cells proliferation, migration and sprouting to enhance new blood vessels formation[15]. After treated with L-arginine and vitamin C, the expression of VEGF was markedly upregulated compared to reduction elicited by high glucose, indicating that L-arginine with vitamin C co-administration was potential in normalizing VEGF-derived angiogenesis and capillary formation impaired by high glucose. Notably, the effect of incubation with L-arginine or vitamin C alone on EC angiogenesis was not so obvious as that in united application.

The mechanism of L-arginine with vitamin C co-treatment in protecting against hypo-angiogenesis injury in T2DM is summarized in Fig. 2. It is well-acknowledged that NO plays an integral role of endothelial homeostasis, mediating the downstream VEGF activity in angiogenesis[19, 29]. As a critical mediator of angiogenesis, NO is mostly produced by NO synthase (NOS) isoforms eNOS and iNOS in cardiovascular system. It has been documented that eNOS plays a pivotal role in NO bioavailability, as it provides primary endogenous amount of NO[7], whereas iNOS acts mainly as a cytotoxic induced by oxidative stress and inflammatory conditions[45]. Previous researches[21, 30, 38] have proved that PI3K/AKT/eNOS signaling pathway actively regulates VEGF-induced neovascularization. Since L-arginine is a substrate for eNOS that produces NO and vitamin C acts as a partially mediator of NO activation, we

continued to investigate whether NOS/NO signaling was the potential molecular mechanisms of VEGF-induced angiogenic regulation of L-arginine with vitamin C co-administration. In this study, by detecting supernatant NO level in RAECs, phosphorylated-eNOS, total eNOS, iNOS protein expression and supernatant content, we demonstrated that combination treatment of L-arginine with vitamin C indeed stimulated NO production likely attributed to eNOS phosphorylation motivation and iNOS inhibition. Additionally, abolishment of eNOS, iNOS and total NOS pointed towards a fundamental role of NOS/NO within angiogenetic process. Taken together, these observations suggest that the hyperglycemia-induced angiogenesis deficiency is in part due to a derangement of the NOS/NO pathway, whereas co-treatment of L-arginine and vitamin C could play a pro-angiogenic role by simultaneously activating eNOS and inhibiting iNOS.

It is well-accepted that the presence of hyperglycemia in diabetes is associated with oxidative stress stimulation, aggravates myocardial ischemia injury. It has been reported[49] that by eliminating O2-, oxidative stress stimulated ROS and RNS accumulation largely resulted from DM, and in turn, interrupts normal metabolism and slows down angiogenesis speed in myocardium[35]. Meanwhile, it was also demonstrated that NOS activity is implicated with over-produced O2-, namely eNOS inactivation and iNOS activation.

A family of NADPH oxidases is regarded as the predominant source of O2-, which disturbs angiogenesis process in both cultured cells and in vivo models of neovascularization[17]. Given the potential therapeutic mechanism of combination therapy, we speculated that though L-arginine appears to be beneficial to reducing oxidative stress on vascular endothelial function in cardiovascular diseases, this effect is modest and hard to work especially at physiological states.

Vitamin C, an antioxidant that scavenges ROS and depletes the excessive accumulation of O2-, may restore NOS/NO pathway activity to promote angiogenesis. This possibility seems to be confirmed by the present study by observing oxidative stress level in vitro. In addition, we verified that NADPH-derived O2- generation is probably the main mechanism responsible for inactivating downstream NO-derived angiogenesis in diabetes. In the context of L-arginine plus vitamin C, this study supports the concept that the amplification of angiogenesis in this combination treatment is associated with the fact that, L-arginine mainly plays the pro-angiogenic role by ameliorating the expression of eNOS, iNOS and NO, ultimately enhancing collateral growth. However, this effect can be partially reduced resulting from oxidative stress. In the capacity of vitamin C as an antioxidant, it lowered the amount of NADPH-derived O2- generation to preserve activation of angiogenic eNOS/NO signaling pathway, amplifying the beneficial effect of L-arginine.

It has been understood that when suffering external damage like hyperglycemia, O2- production can rapidly react with NO, resulting in the generation of peroxynitrite (ONOO-)[8, 34], under myocardial injury. The production of ONOO- increased by DM-induce NADPH is an intense cytotoxic oxidant that in turn, deteriorates stress states and aggravates downstream angiogenesis in diabetic myocardial injury. Alternatively, ONOO- increases further predisposes the heart to NOS uncoupling, which is associated with

eNOS inactivation. According to previous findings, excessive exogenous L-arginine supplement may shift to an injurious scenario with increased production of ONOO-. Nevertheless, ONOO- generation can be abrogated by vitamin C due to the effect on O2- scavenging. Similar conclusions have been reached in the present study, in which co-supplementation of L-arginine and vitamin C lessened the level of ONOO- and increased NO bioavailability. This notion explains the phenomenon that even treated with vitamin C alone, it mildly activated eNOS activity. Therefore, another advantage of the combination application of L-arginine with vitamin C is, oral administration of vitamin C fills the gap of L-arginine that produces ONOO- to decrease adverse reactions.

It may be worth noting that apart from the pharmacodynamics interactions, the mutual effects on pharmacokinetics are deserved further assessment. We focus our interest on the status of intra-cellular arginine metabolism in affecting endothelial angiogenesis, more specifically, governing eNOS activity and NO production. It has been reported that as the predominant contributor among different arginine transporters in endothelial cells, cationic amino acid transporter 1 (CAT-1) can be modulated by multiply redox-sensitive mechanisms[26]. Interestingly, Schwartz et al[39] has elaborated the underlying interaction of CAT-1 with eNOS activity that CAT-1 specially delivers transported arginine to membrane-bound eNOS. Thus, in our study, we detected the effect of high glucose stimulation combination treatment of L-arginine with vitamin C on arginine uptake by measuring CAT-1 expression in RAECs. The result suggested that when combined with vitamin C, exogenous L-arginine was transported into cells more active compared with L-arginine single treatment, suggesting that vitamin C improved the availability and utilization efficiency of L-arginine. Nevertheless, incubation with vitamin C alone showed limited benefits possibly attributed to the extra-cellular L-arginine concentrations. To this end, this described pharmaceutical interactions may pave the way for the potential amplified therapeutic effect of this promising combined therapy.

In addition, the therapeutic effects and mechanisms of L-arginine plus vitamin C must be examined in vivo experiments. In our study, AMI rats under the condition of T2DM impaired collateral formation in myocardium, providing a well-recognized model of angiogenesis deficiency. By the changes in biochemical criterions, we found that L-arginine with vitamin C co-administration inhibited the level of blood lipid and decreased the blood glucose in T2DM injury. The exogenous administration of L-arginine with vitamin C improved cardiac function by the detection of LVEF and LVFS, along with the result of LDH serum level. Nevertheless, the application of L-arginine or vitamin C alone has not induced significant effect of improved cardiac function, which is in agreement with results in vitro. What's more, the relaxation of aortic rings responding to endothelium-dependent vasodilator such as acetylcholine but not to an endothelium-independent vasodilator such as SNP was impaired in T2DM rats as well as T2DM + AMI rats, whereas the impairment could be attenuate by L-arginine and vitamin C co-intervention. As impaired vasoconstrictor responses to phenylephrine were restored by treatment of aortic rings with L-arginine and L-NNA, this implies that basal and inducible NO production is enhanced in L-arginine plus vitamin C group. In line with the in vitro ratiocinations, by observing the results of MVD assay, VEGF immunohistochemistry and TTC staining, we concluded that L-arginine + vitamin C co-supplementation reduced infarction size, promoted angiogenesis and arteriogenesis in DM-induced impairment of

coronary collateral formation, showing an amplify effects compared with single treatment. At the same time, the results from plasma NO levels, eNOS and iNOS immunohistochemistry as well as their serum contents, can further prove findings in vitro that a functional association between L-arginine and vitamin C combined therapy and NOS/NO-induced angiogenesis. Our in vivo results also supported the in vitro data showing that L-arginine and vitamin C co-treatment decreased MDA serum level and reduced NADPH expression, confirming the therapeutic mechanism of NADPH-derived oxidative stress inhibition.

Endothelial cell angiogenesis contributes to endothelial function impairment resulting in vascular dysfunction T2DM. Thus, to further verify the role of L-arginine and vitamin C in response to vascular dysfunction induced by diabetic AMI, vasodilation test was assayed in rats. As endothelial function is determined by Ach-induced relaxation, we monitored Ach-induced endothelium-dependent vasodilatation. As indicated in Fig. 9a-c, hyperglycemia impaired endothelium-dependent relaxation obviously in T2DM and T2DM + AMI group. After 2 weeks treatment of L-arginine and vitamin C, the reduction of Ach-induced vasodilatation was been reversed. According to the analytical data, combination therapy enhanced the endothelium-dependent relaxation more significantly in T2DM rats than that treated L-arginine or vitamin C alone. Additionally, vascular response to SNP, a NO donor, was tested, which evoked endothelium-independent vasodilatation in thoracic aorta. By measuring endothelium-independent relaxation, it can be inferred that the normal function of vascular smooth muscle cells didn't been damaged in terms of NO.

As NO plays the vital role of endothelial function, NO release under basal and inducible conditions were also detected. The concentration-response curves to phenylephrine on the aortic rings among 6 groups were showed in Fig. 9d-g. Pretreatment with L-Arginine, the NO synthesis substrate, weakened the concentration-response to phenylephrine in the endothelium-intact aortic rings. On the contrary, after the incubation with the NOS inhibitor L-NNA, the contractile response to phenylephrine was strengthened. As illustrated in, T2DM as well as T2DM + AMI rats showed reduced ΔAUC. Under the influence of L-arginine and vitamin C co-treatment, the decreased ΔAUC was reversed, implying that combination treatment availably restored hyperglycemia-impaired NO basal release and induced release. Taken together, we testified that co-application of L-arginine and vitamin C ameliorated cardiac trauma together with vascular dysfunction of diabetic AMI rats.

Conclusion

In conclusion, we found the pro-angiogenic property of L-arginine and vitamin C combination treatment, which is promising to be a potential angiogenesis therapeutic strategy. According to the results in this study, we demonstrated that DM elicited a diminished angiogenic response to myocardial ischemia, which could be normalized by co-treatment of L-arginine and vitamin C in vivo and in vitro. Additionally, this combined application mainly exerts a more pronounced, synergistic protective function on angiogenesis recovery and concomitant vascular and cardiac function compared with that of exogenous L-arginine or vitamin C administrations alone. The molecular mechanism seems to be associated with inhibition of NADPH-induced O_2^- generation, following with the downstream NOS/NO/VEGF signaling pathway restoration. Namely, L-arginine with mildly attenuating oxidative stress resulted in activating

eNOS and inactivating iNOS, ultimately supplying NO to faster angiogenesis, simultaneously, the addition of vitamin C amplified the antioxidative effect on multiply damages and enhanced the availability of L-arginine. However, further studies are required to elucidate deeper molecular basis underlying the efficacy of L-arginine plus vitamin C, especially the interaction of physiological disposition.

Abbreviations

AMI: acute myocardial infarction; GSH-PX: glutathion peroxidase; LAD: left anterior descending artery; L-ARG: l-arginine; LVEDD: left ventricular end diastolic diameter; LVEDV: Left ventricular end diastolic volume; LVEF: left ventricular ejection fraction; LVFS: left ventricular fraction shortening; LVESD: left ventricular end systolic diameter; LVESV: left ventricular end systolic volume; NO: nitric oxide; NOS: nitric oxide synthase

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JS performed experiments, analyzed data and wrote draft. BBC carried out the molecular studies and assisted in the analysis of data. XXL assisted in the animal experiments and performed experiments. MX supervised the study, conceived of the study, participated in its design and drafting of the manuscript. All the authors read and approved the final manuscript.

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Figures

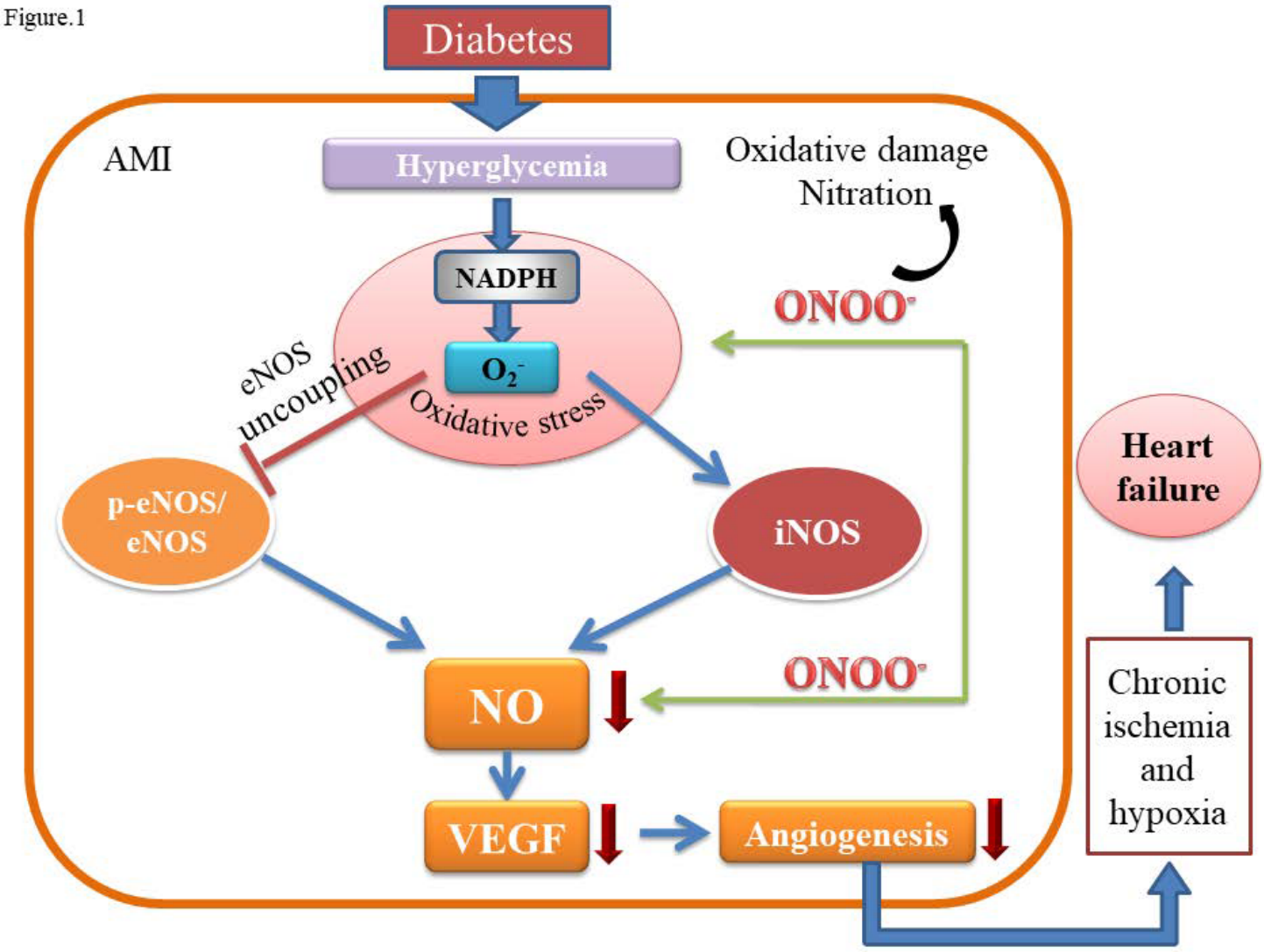

Figure 1

Diabetes mellitus impairs the ability of angiogenesis in ischemic hearts leading to low speed restoration of myocardium perfusion. Hyperglycemia, the main cause of overproduction of reactive oxygen species (ROS), accumulation of O2- may be involved in the inhibition of the vascular repair mechanisms through NOS/NO signaling injury. The growing NADPH oxidases activates O2- generation, then inhibits eNOS activity as well as iNOS activation. The change of NOS production directly depresses NO production. To this end, VEGF-induced neovascularization is restrained, which caused insufficient collateral perfusion, eventually caused chronic ischemia and hypoxia in diabetic hearts.

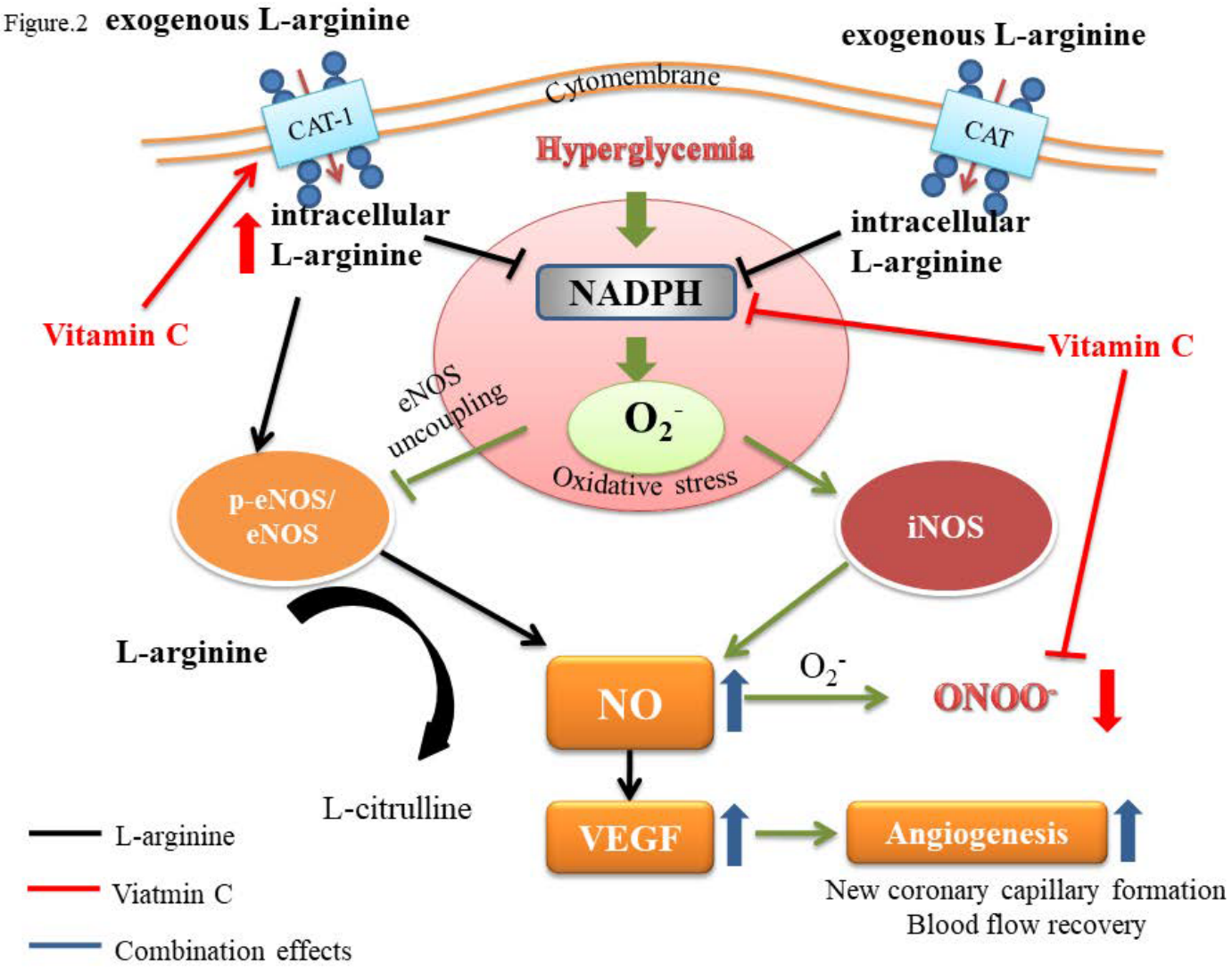

Figure 2

Putative mechanisms of co-administration of L-arginine with vitamin C on angiogenesis in diabetic hearts. Hyperglycemia causes an increase of ROS generation which suggests downstream NO production block, following with VEGF inhibition. With the help of L-arginine with vitamin C application, high glucose-induced angiogenesis damage was inhibited by decreasing O2- production and ROS production through NADPH oxidases inactivation but also by up-regulating NOS/NO signaling. This treatment effectively activates eNOS activity, whereas reduces iNOS production. By regulating NOS/NO signaling pathway, damaged angiogenesis ischemic myocardium is ameliorated. In addition to interfering NOS/NO/VEGF induced angiogenesis, the combination of these two drugs shows better outcomings partially explained by the NO bioavailability and L-arginine uptake increase due to vitamin C.

Figure.3

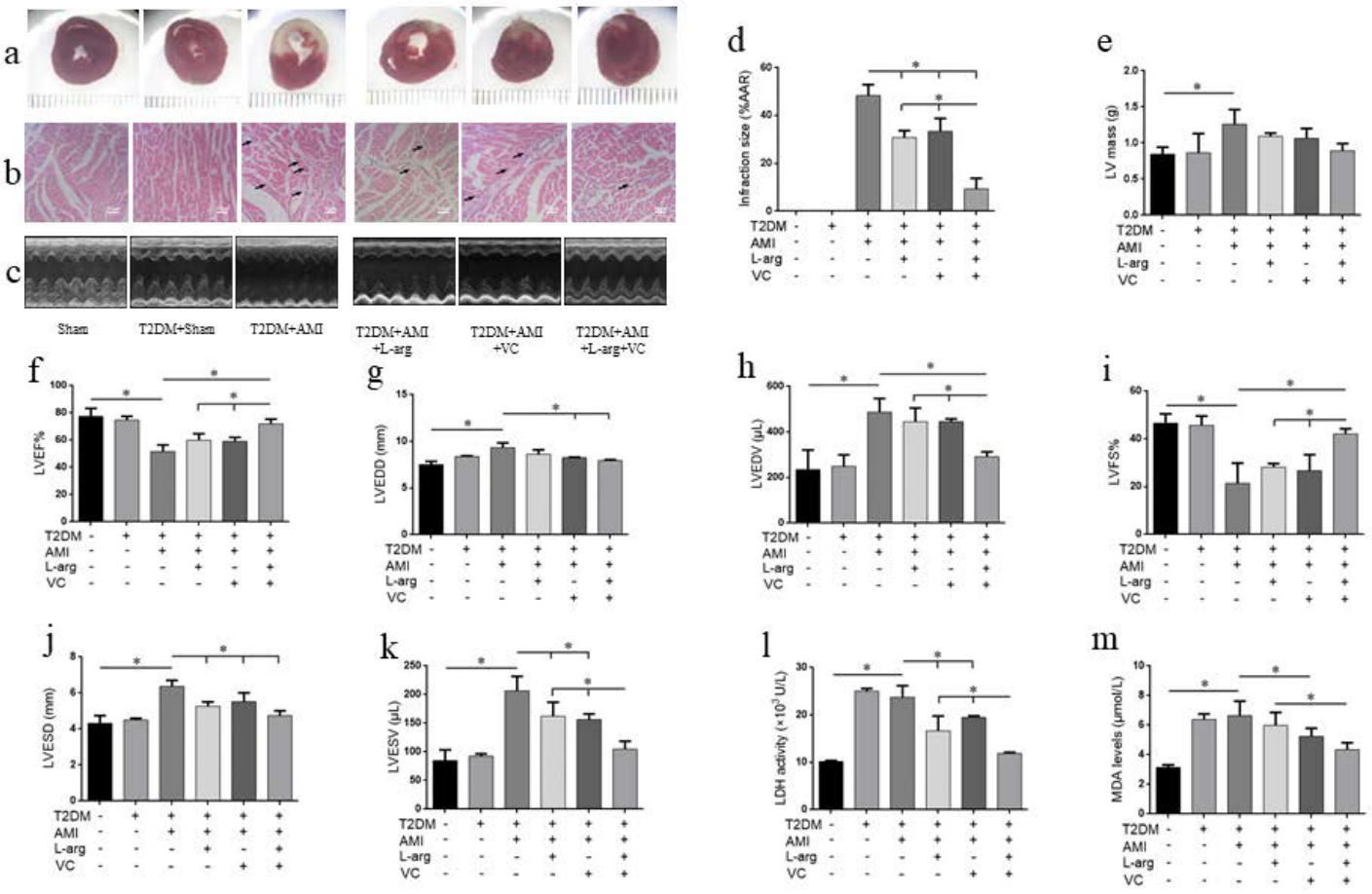

Figure 3

Co-treatment of L-arginine and vitamin C ameliorates cardiac function. T2DM rats were subjected to AMI injury as detailed in the Methods. Representative photographs (a) of TTC stained sections from perfused hearts. Infarct size (d) were quantified for L-arginine and vitamin C treated T2DM +AMI group and no treatment T2DM+AMI rats. Representative photographs (b) of HE staining were shown as the myocardial injury. Representative M-mode echocardiograms (c) of the parasternal short-axis view obtained in each group after AMI operation. Summarized data of LV mass (e), LVEF (f), LVEDD (g), LVEDV (h), LVFS (i), LVESD (j), LVESV (k) was exhibited to show the changes of left ventricle in each group. Summarized data of LDH activity (l) and MDA levels (m) showed the degree of cardiac damage. *P < 0.05 (n = 4)

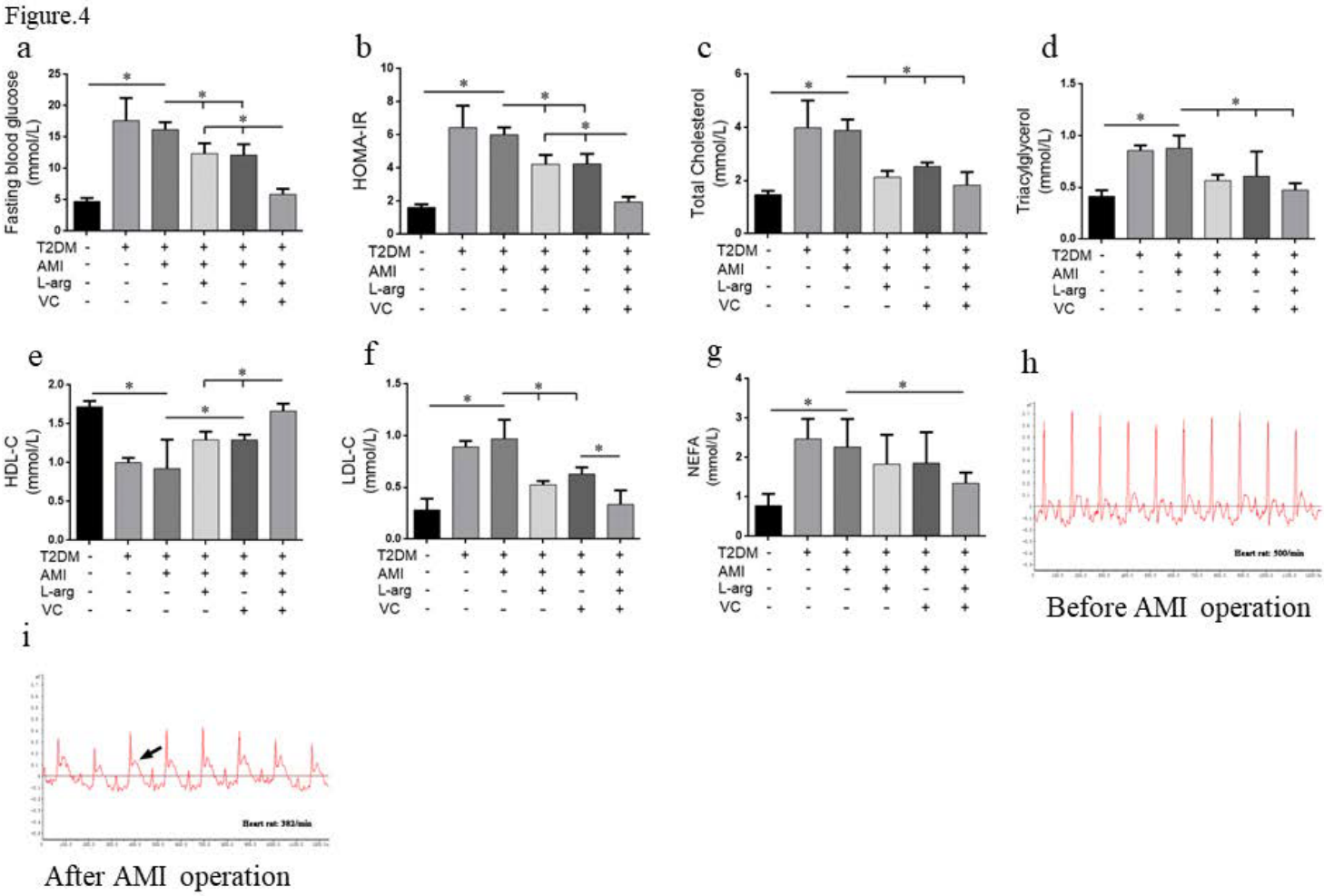

Figure 4

L-arginine with vitamin C treatment inhibits insulin resistance in T2DM rats. Summarized data showed the FBG (a), HOMA-IR (b), TC (c), TG (d), HDL-C (e), LDL-C (f) and NEFA (g). Representative ECG images before (h) and after (i) AMI operations showed the development of AMI models. *P < 0.05 (n = 6)

Figure.5

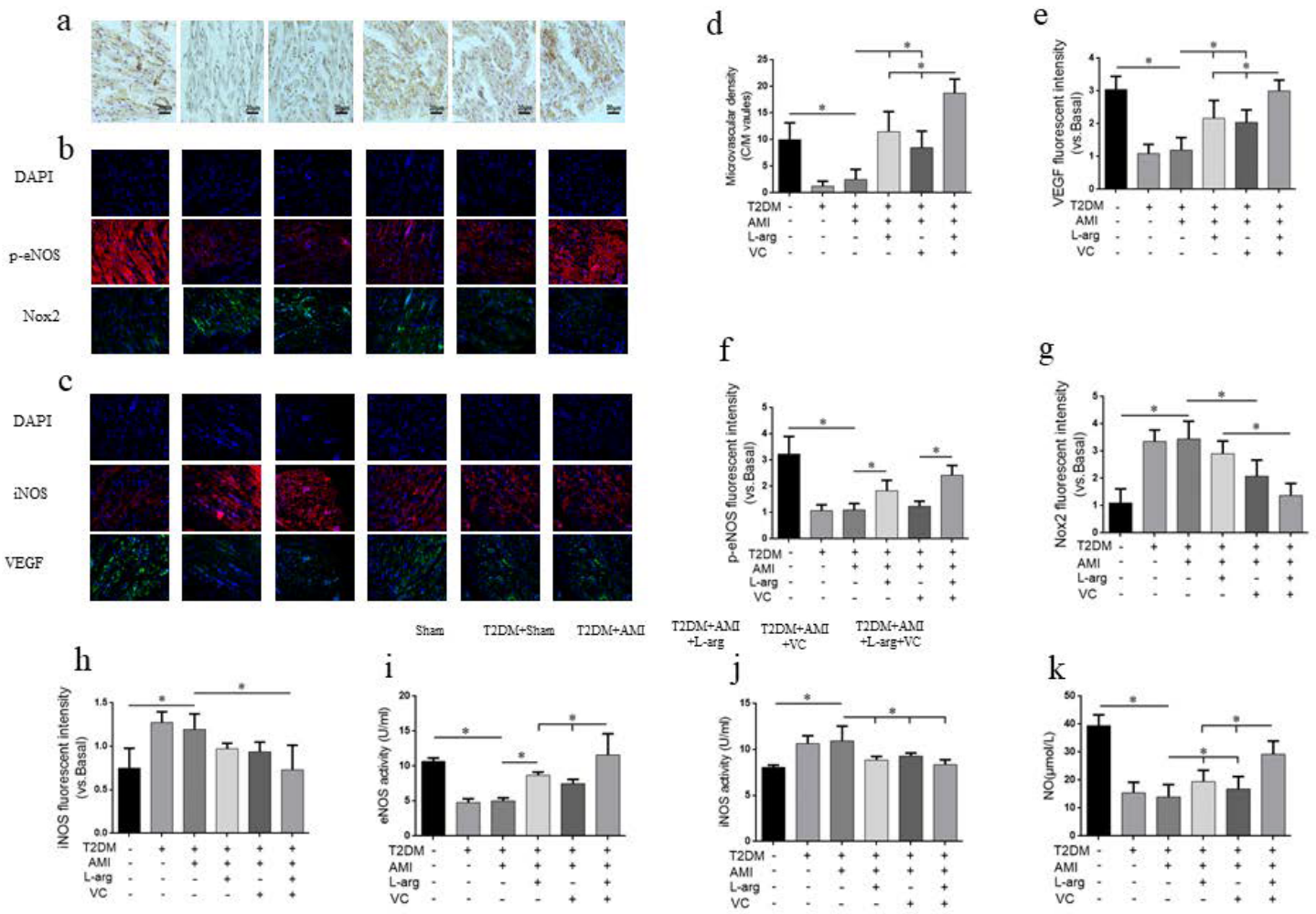

Figure 5

L-arginine with vitamin C application improved NOS/NO induced angiogenesis in diabetic AMI rats. Representative photographs (a) of capillary density in ischemic myocardium at 2 weeks after L-arginine and vitamin C treatment. (d) Quantification of capillary density in ischemic myocardium after medicine administration. Double immunofluorescence staining (b, c) and relative fluorescence values (e, f, g, h) illustrating myocardial levels of VEGF, p-eNOS, Nox2 and iNOS in T2DM rats Summarized data of eNOS (i), iNOS (j), NO release (k) in the serum. *P < 0.05 (n = 6)

Figure.6

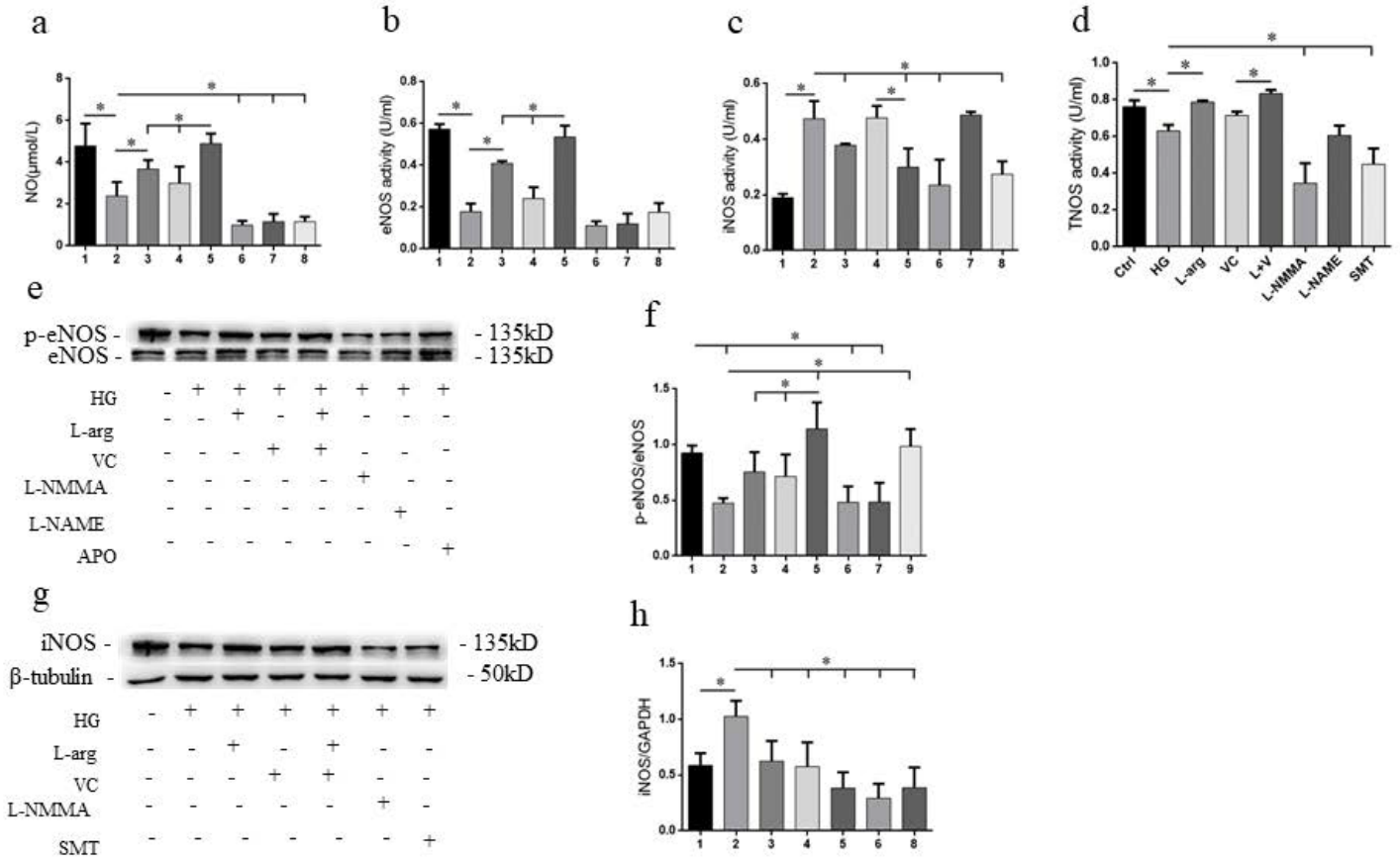

Figure 6

Co-treatment of L-arginine and vitamin C facilitates eNOS-dependent NO production and blocks iNOS-dependent NO production. Summarized data showed the role of L-arginine and vitamin C on NO production (a), eNOS (b), iNOS (c), and tNOS (d) expression, in the RAEC supernatant. Representative Western blot gel documents (e) and summarized data (f) showed the effects of L-arginine with vitamin C on the protein expression of p-eNOS and eNOS. Similarly, iNOS expression was shown in the representative western blot gel documents (g) and summarized data (h). *P < 0.05 (n = 4)

Figure.7

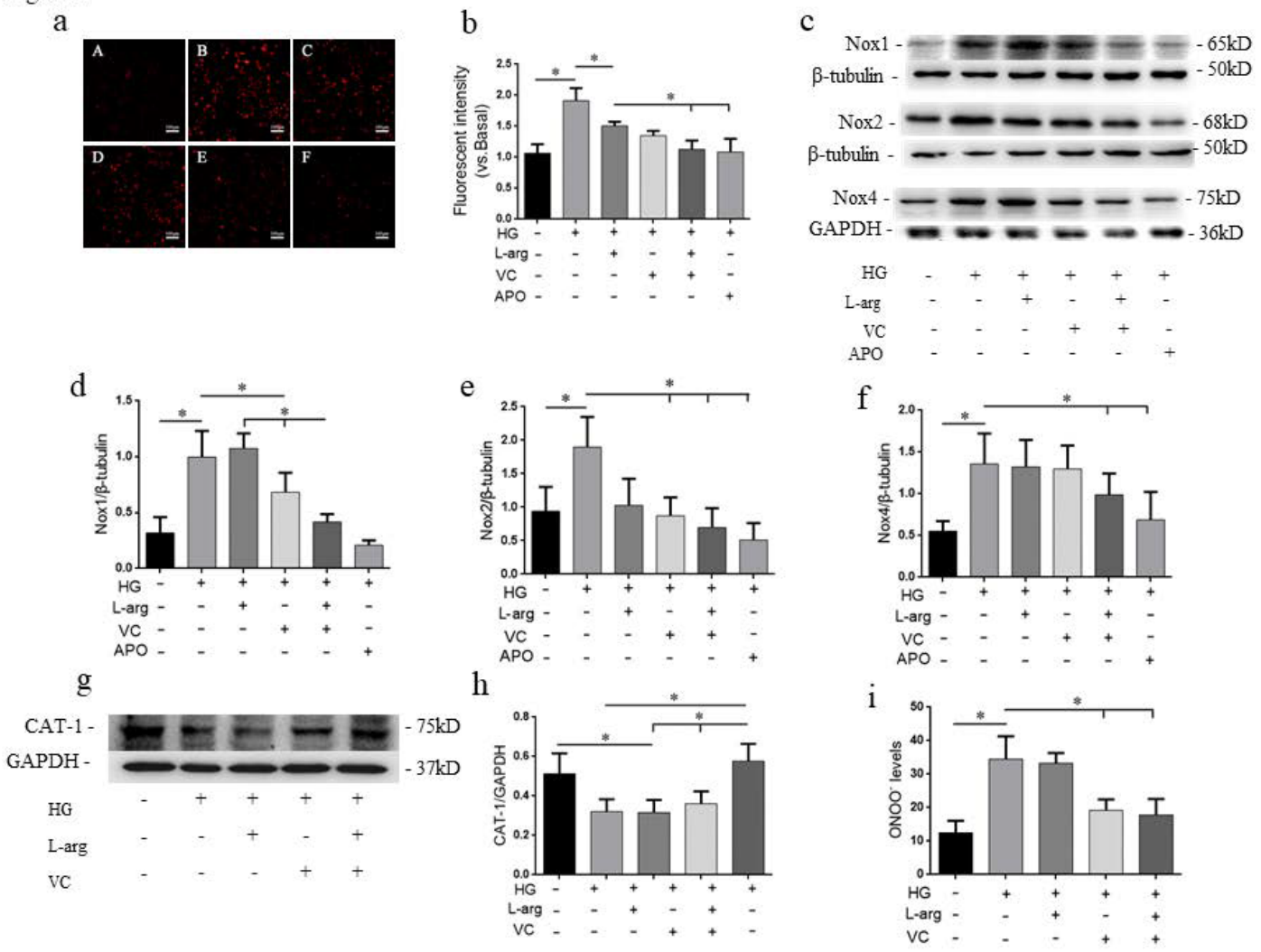

Figure 7

Co-administration of L-arginine and vitamin C decreased NADPH oxidase-induced intracellular O2- generation in RAECs. Typical representative fluorescent images for DHE staining (a) and summarized fluorescent intensity (b) showed the effects of L-arginine, vitamin C and APO on O2- production in RAECs incubated with high glucose. Representative Western blot gel documents and summarized data showed the role of this combined treatment on Nox1 (c,d), Nox2 (e,f) and Nox4 (g,h) expression. Representative Western blot gel documents (g) and summarized data (h) showed the effect of this combined treatment on CAT-1. Summarized data (i) of ONOO- level in RAECs was demonstrated to clarify the benefits of combined application. *P < 0.05 (n = 4)

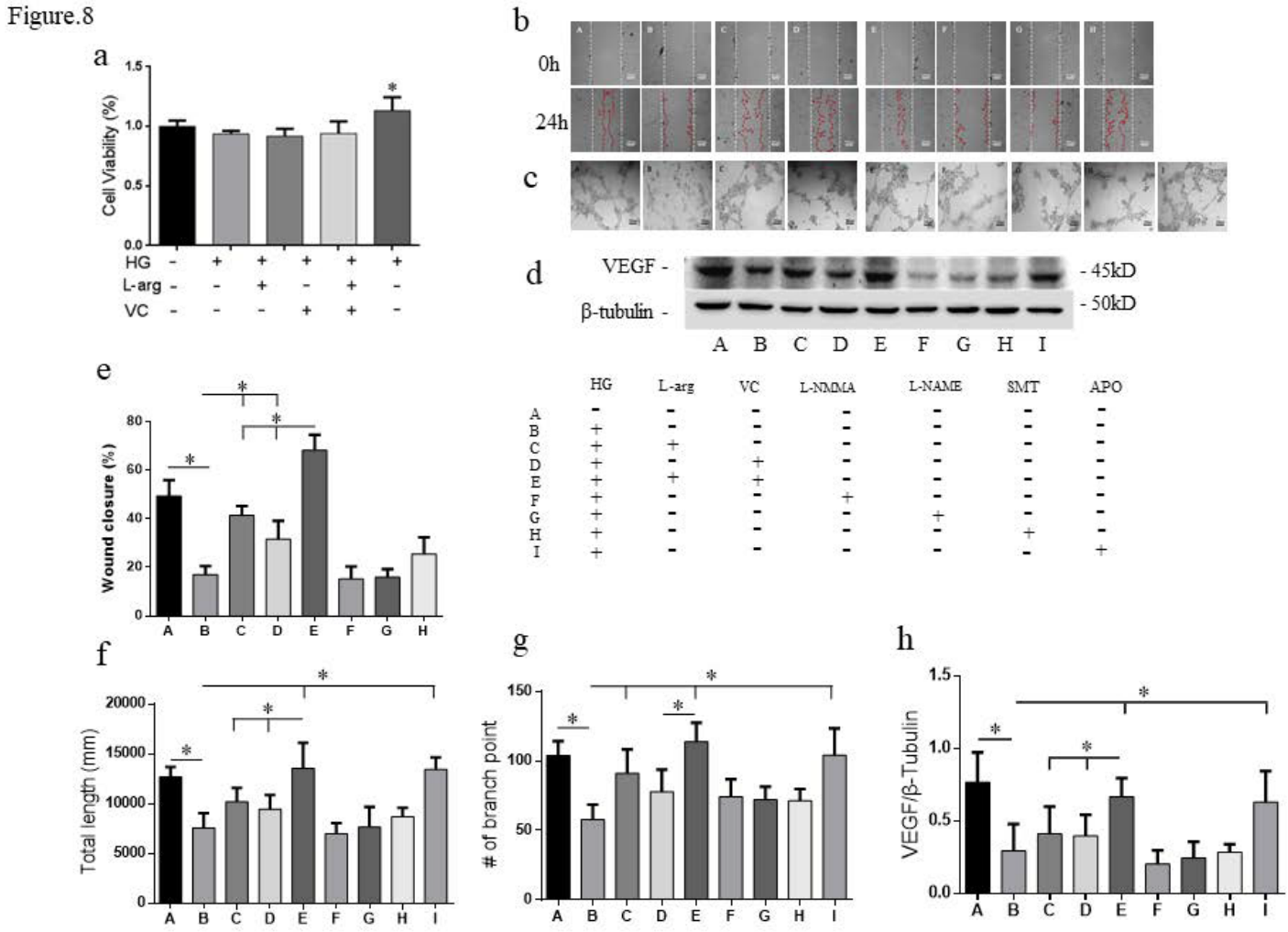

Figure 8

Co-treatment of L-arginine and vitamin C improved angiogenesis in RAECs. Summarized data (a) showing the high glucose-induced cell viability under the treatment of L-arginine and vitamin C alone or together. Representative photograph (b) of RAEC migration after culturing with high glucose, L-arginine, vitamin C, alone or co-incubation, and L-NMMA, L-NAME, SMT. Bars: 200μm. (e) Quantification of cell migration. Scratch wound closure assays revealed that co-treatment of L-arginine and vitamin C significantly improves cell migration. (c) Representative photograph of RAEC Matrigel tube formation after culturing with L-arginine, vitamin C and three inhibitors. (f, g) Quantification of Matrigel tube formation. The tube length and branching point numbers were significantly higher in the co-treatment group as compared to the L-arginine or vitamin C single use, almost the same effect as NADPH inhibitor, APO. Bars: 200μm. (d) Representative western blot gel documents showing the protein expression of VEGF. Summarized data (h) showing the VEGF protein expression of RAECs treated with different culture medium. *P < 0.05 (n=4)

Figure.9

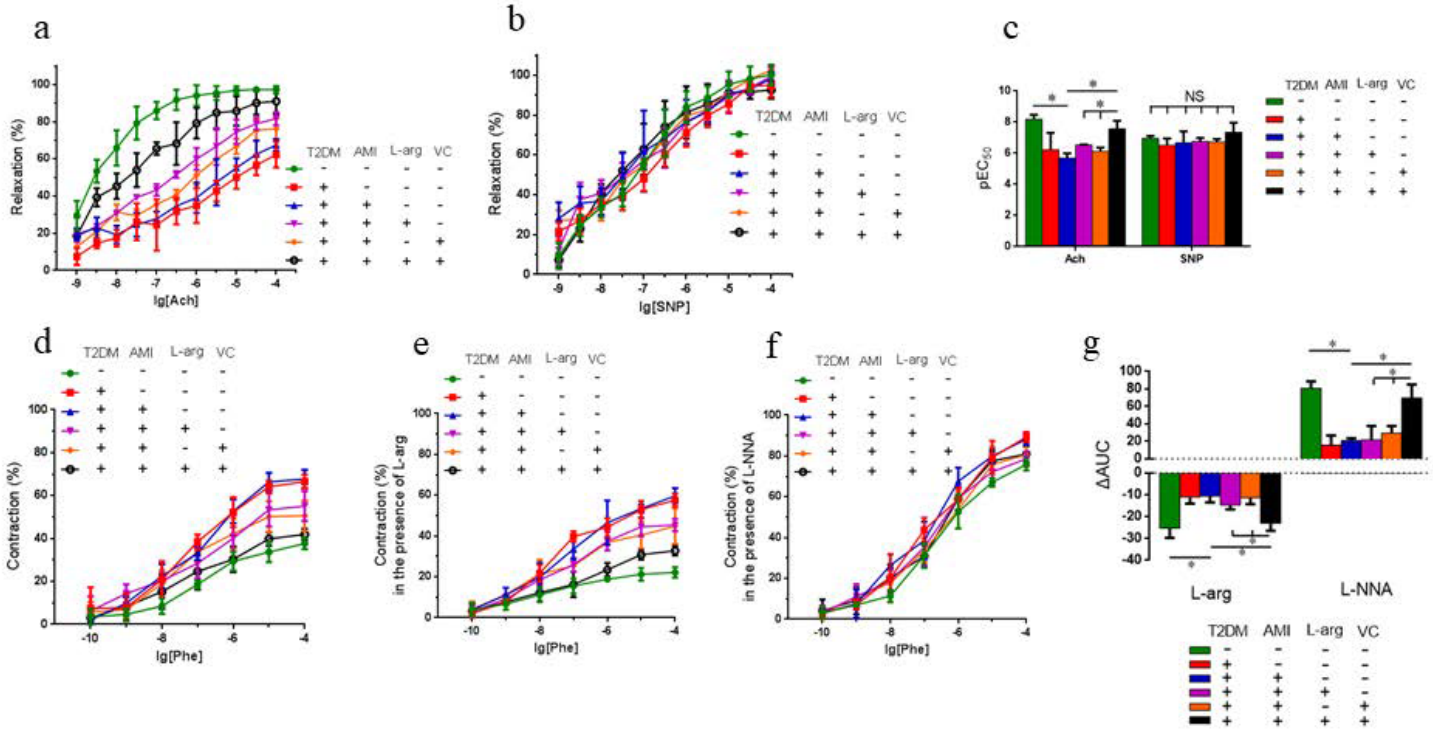

Figure 9

Combined use of L-arginine and vitamin C ameliorated vascular dysfunction in diabetic AMI rats. Vascular relaxant response to acetylcholine (Ach, 10-10–10-4M) and sodium nitroprusside (SNP,10-10–10-4M) were determined on thoracic aorta from diabetic rats (a, b). summarized data (c) showed the role of L-arginine and vitamin C co-administration on the pEC50 to Ach and SNP. Vascular concentration-response curves to phenylephrine (Phe) in the absence (d) and presence of L-Arginine (L-Arg, 10-4M) (e) or L-NG-Nitroarginine (L-NNA, 10-4 M) (f) were determined in thoracic aorta, respectively. ΔAUC of the contractile response to Phe represented the change of induced NO release and basal NO level (g). *P < 0.05 (n=4)