Original Research

Beetroot juice reduces infarct size and improves cardiac function following ischemia–reperfusion injury: Possible involvement of endogenous H_2S

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Abstract

Ingestion of high dietary nitrate in the form of beetroot juice (BRJ) has been shown to exert antihypertensive effects in humans through increasing cyclic guanosine monophosphate (cGMP) levels. Since enhanced cGMP protects against myocardial ischemia–reperfusion (I/R) injury through upregulation of hydrogen sulfide (H_2S), we tested the hypothesis that BRJ protects against I/R injury via H_2S. Adult male CD-1 mice received either regular drinking water or those dissolved with BRJ powder (10 g/L, containing ~0.7 mM nitrate). Seven days later, the hearts were explanted for molecular analyses. Subsets of mice were subjected to I/R injury by occlusion of the left coronary artery for 30 min and reperfusion for 24 h. A specific inhibitor of H_2S producing enzyme – cystathionine-γ-lyase (CSE), DL-propargylglycine (PAG, 50 mg/kg) was given i.p. 30 min before ischemia. Myocardial infarct size was significantly reduced in BRJ-fed mice ($15.8 \pm 3.2\%$) versus controls ($46.5 \pm 3.5\%$, mean ± standard error [*SE*], $n = 6$/group, $P < .05$). PAG completely blocked the infarct-limiting effect of BRJ. Moreover, BRJ significantly preserved ventricular function following I/R. Myocardial levels of H_2S and its putative protein target – vascular endothelial growth factor receptor 2 (VEGFR2) were significantly increased by BRJ intake, whereas CSE mRNA and protein content did not change. Interestingly, the BRJ-induced cardioprotection was not associated with elevated blood nitrate–nitrite levels following I/R nor induction of cardiac peroxiredoxin 5, a mitochondrial antioxidant enzyme previously linked to nitrate-induced cardioprotection. We conclude that BRJ ingestion protects against post-I/R myocardial infarction and ventricular dysfunction possibly through CSE-mediated endogenous H_2S generation. BRJ could be a promising natural and inexpensive nutraceutical supplement to reduce cardiac I/R injury in patients.

Keywords: Natural dietary supplement, cardioprotection, ischemia–reperfusion injury, myocardial infarction, nitrate, H_2S

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Introduction

Ischemic heart disease remains the number 1 killer worldwide according to the World Health Organization (http://www.who.int/mediacentre/factsheets/fs310/en/index.html). Although considerable progress has been made toward identifying pharmacological agents that are capable of protecting heart against ischemia–reperfusion (I/R) injury, none of these agents was without limitations and substantial side effects and routinely used in the cardiology clinics. Therefore, novel, safe, and cost-effective therapeutic strategies are urgently needed for combating against myocardial I/R injury. In this regard, a major nutraceutical initiative has been to increase public awareness of vegetable consumption in an effort to reduce cardiovascular disease.[1] This approach is mainly based on epidemiological,[2] cohort,[3,4] and trial-based[5,6] data demonstrating that increased consumption of a vegetable-rich diet limits the incidence of cardiovascular disease. Recent studies have suggested that the cardiovascular beneficial effects of vegetables are related to the high content of inorganic nitrate, which in concert with symbiotic bacteria in the oral cavity is converted into nitrite, nitric oxide (NO), and its metabolites that possess vasodilating and tissue-protective properties.[7] Dietary nitrate clearly has robust NO-like effects in humans, including the reduction of blood pressure, inhibition of platelet aggregation, and vasoprotective activity.[8,9] This protective mechanism of nitrate is especially important

under various pathological conditions such as I/R injury, which is linked to oxidative stress and reduced NO bioavailability. In animal or human subjects, dietary nitrate protects against I/R injury[10] and other types of cardiovascular disorders such as peripheral arterial disease[11] and pulmonary hypertension.[12] In addition, we recently demonstrated that oral nitrate supplementation protects against doxorubicin-induced cardiomyopathy by improving mitochondrial function.[13,14]

Beetroot juice (BRJ) is one of the highest nitrate-containing vegetable products. The consumption of BRJ exerts beneficial effects in healthy volunteers, including lowering of both systolic and diastolic blood pressures, through elevation of cyclic guanosine monophosphate (cGMP) concentration, and protection of the endothelium from I/R-induced endothelial damage.[15] The cGMP-dependent signaling pathway is particularly important in cardioprotection because several studies from our laboratory showed that pharmacological inhibition of cGMP-specific phosphodiesterase-5 (PDE-5) with sildenafil or vardenafil exert powerful protective effects against I/R injury[16–18] and heart failure[19,20] likely via activation of NO-dependent signaling pathways.[17,21] Recently, we also showed that the long-acting PDE-5 inhibitor, tadalafil, protects the heart against I/R injury through protein kinase G (PKG) -mediated generation of hydrogen sulfide (H_2S), and this protection was lost in the mice deficient of cystathionine-γ-lyase (CSE) – a H_2S-producing enzyme.[22] Considering the well-demonstrated beneficial effects of BRJ in hypertension and vascular endothelial dysfunction,[15] as well as liver I/R injury,[23] we undertook the present study to test a novel hypothesis that BRJ protects against myocardial I/R injury through generation of H_2S. Along with the physiological studies investigating the effects of BRJ ingestion on post-I/R myocardial infarct size and ventricular function, we also examined mRNA and protein levels of two main H_2S-producing enzymes, CSE and cystathionine β-synthase (CBS), in the heart tissues. In addition, peroxiredoxin 5 (Prx5) is a mitochondrial antioxidant enzyme whose cardioprotective role was recently suggested in the settings of inorganic nitrate-alleviated doxorubicin cardiotoxicity.[13,24] The nitrate-induced cardioprotective effects were associated with enhanced plasma NO levels and cardiac expression of Prx5.[14] Therefore, we sought to investigate if there is any increase in NO production and/or Prx5 expression that may contribute to the cardioprotection of BRJ ingestion against I/R injury. The preliminary data of the present study had been presented at the 85th Scientific Sessions of the American Heart Association held at Los Angeles, CA in November 2012.[25] Finally, since VEGFR2 was recently identified as a direct protein target of H_2S,[26,27] we further examined cardiac expression of VEGFR2 in control and BRJ-treated mice.

Materials and methods

Animals

Adult male outbred CD-1 mice were supplied by Charles River Laboratories International, Inc. (Wilmington, MA, USA). The body weight ranged from 28 to 33 g. All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by National Institutes of Health (No. 85–23, revised 1996) and the experimental protocol was approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

Drugs, chemicals, and BRJ

DL-propargylglycine (PAG) and triphenyltetrazolium chloride (TTC) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Certified organic BRJ powder was purchased from either Walther Schoenenberger Pflanzensaftwerk GmbH & Co. (Magstadt, Germany) or PINES International, Inc. (Lawrence, KS, USA). These BRJ products were chosen solely because of their immediate availability from the dietary supplements stores during the initial and continuation stages of the current study. BRJ was prepared by dissolving 10 g of BRJ powder per 1 L of drinking water. The freshly prepared BRJ samples were collected for measuring its nitrate contents with a NO analyzer (see *STUDY 3* subsequently for more details), which consistently indicated a level of 0.7-mM nitrate.

Experimental groups and protocols

As illustrated with details in Figure 1, total of 82 male CD-1 mice were used for this investigation and randomly assigned to the following series of five substudies, which are described, respectively, subsequently.

STUDY 1 on myocardial infarct size (24 mice in four groups). The animals were randomized into the following four treatment groups ($n = 6$/group): Group 1 (*Control*): Mice received regular drinking water without any treatment prior to I/R; Group 2 (*BRJ*): Mice received BRJ for seven days prior to I/R; Group 3 (*BRJ + PAG*): BRJ-fed mice (similar to Group 2) were administered with PAG (PAG, a CSE inhibitor, 50 mg/kg, i.p.) 30 min before I/R; and Group 4 (*PAG*): PAG alone was given to the nonpretreated mice 30 min prior to I/R. Following an established *in vivo* protocol of cardiac I/R injury,[22] all of the four groups of mice were subjected to 30 min of regional ischemia through occlusion of the left descending coronary artery and the subsequent reperfusion for 24 h (Figure 1). At the end of reperfusion, the heart was quickly removed and mounted on a Langendorff apparatus. The coronary arteries were perfused with 0.9% NaCl containing 2.5-mM $CaCl_2$. After the blood was washed out, ~1 mL of 10% Phthalo blue dye was injected as a bolus into the aorta until most of the heart turned blue. The heart was perfused with saline to wash out the excess Phthalo blue. Finally, the heart was removed, frozen, and cut into 8–10 transverse slices from apex to base of equal thickness (~1 mm). The slices were then incubated in 10% TTC dissolved in isotonic phosphate buffer (pH 7.4) for 30 min at room temperature. The areas of infarcted tissue, the risk zone, and the entire left ventricle were measured by computer morphometry using a BIOQUANT image analysis software (Nashville, TN, USA).

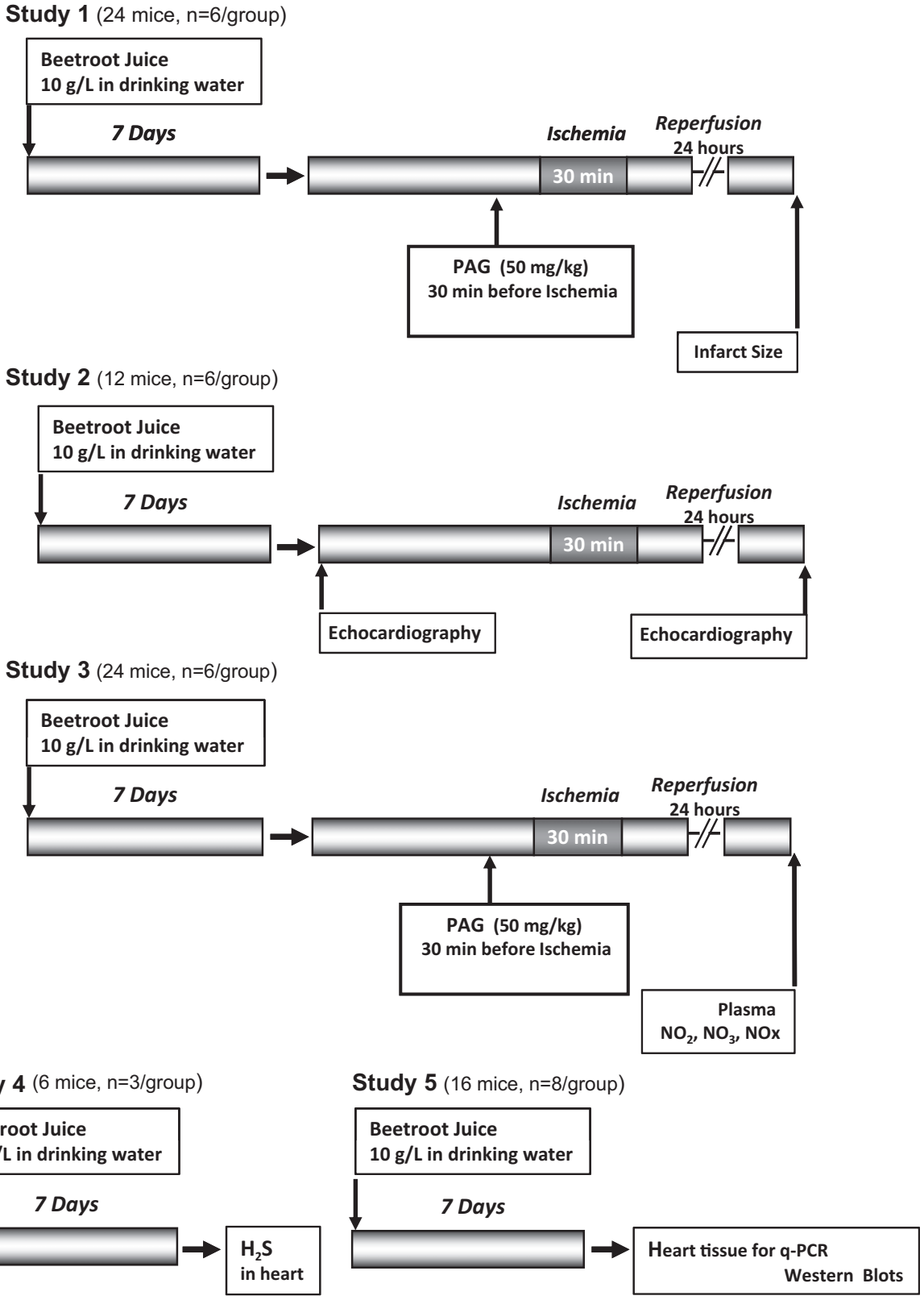

Figure 1 Experimental protocols for *in vivo* surgeries and collection of blood and heart tissue samples. Arrows indicate time points for treatment, performance of surgical procedures, and measurement of various parameters. PAG is abbreviated term of DL-propargylglycine – a specific inhibitor of H_2S producing enzyme – cystathionine-γ-lyase (CSE)

STUDY 2 on left ventricular contractile function (12 mice in two groups). Similar to the above-mentioned Groups 1 and 2, a subset of mice ($n = 6$/group) ingested regular water (Control) or BRJ for seven days prior to the same I/R protocol (Figure 1). Echocardiography was performed using the Vevo770™ imaging system (VisualSonics Inc., Toronto, Canada) prior to surgery (i.e. the composite baseline for both control and BRJ groups, $n = 12$) and at the end of 24 h reperfusion ($n = 6$/group) prior to sacrificing the animals. Under light anesthesia with pentobarbital (30 mg/kg; i.p.), the procedure was carried out to measure left ventricular (LV) end-diastolic diameter (LVEDD), end-systolic diameter (LVESD), anterior wall diastolic thickness (AWDT), and posterior wall diastolic thickness (PWDT). LV fractional shortening (FS) was calculated as (LVEDD − LVESD)/LVEDD × 100.[22]

STUDY 3 on plasma nitrate and nitrite levels (24 mice in four groups). As shown in Figure 1, additional 24 mice were assigned into four treatment groups ($n = 6$/group): Group 1 (*Sham*): mice were subjected to a sham surgical protocol that included all the surgical procedures except I/R; Group 2 (*I/R*): mice received regular water and subjected to I/R protocol; Group 3 (*BRJ + I/R*): mice received BRJ for seven days prior to I/R; and Group 4 (*BRJ + I/R + PAG*): BRJ-fed mice were administered with PAG 30 min before I/R. At the end of 24-h reperfusion period, the mice were reanesthetized with pentobarbital (100 mg/kg; i.p.) and their blood samples (1–2 mL per mouse) were collected from the opened thoracic and abdominal cavities and centrifuged at 1250 × *g* to obtain the supernatant plasma. The plasma samples were subsequently centrifuged using Amicon Ultra-4 centrifugal filter devices at 7500 × *g* in 4°C to eliminate large molecules (molecular weight > 30 kDa) from the plasma. The levels of nitrate and nitrite were measured with a SIEVERS NO analyzer (model 280NOA) similar to our previous publication.[13]

STUDY 4 on cardiac tissue levels of H_2S (six mice in two groups). Another subset of six mice was utilized for H_2S measurement. Group 1 was nontreated control and Group 2 received BRJ for seven days prior to heart collection ($n = 3$/group). The tissue concentration of H_2S was measured by homogenizing snap-frozen hearts in 1 mL of 100-mM potassium phosphate buffer (pH 7.4). To trap H_2S, 250 μL of zinc acetate (1% wt/vol) was added to the tissue homogenate followed by 30-min incubation at 37°C. The reaction was stopped by adding 250 μL of trichloroacetic acid (10% wt/vol) to the assay mixture and incubated for 60 min at 37°C before centrifugation at 14,000 × *g* for 10 min. H_2S concentration of the supernatants was measured using a highly specific H_2S sensor connected to an Apollo 1000 free radical analyzer (World Precision Instruments, Sarasota, FL, USA) and was calculated using a calibration curve of NaHS standards. Protein concentration was measured spectrophotometrically at 595 nm. The results are expressed as μM/mg protein.[28]

STUDY 5 on cardiac mRNA and protein expressions (16 mice in two groups). Similar to *STUDY 4*, Group 1 was nontreated control and Group 2 received BRJ for seven days prior to heart collection ($n = 8$/group). The collected cardiac tissue samples were divided half and half for further molecular analyses in determining mRNA and protein expression of several relevant gene–protein targets specified subsequently.

Quantitative PCR

The total RNA was extracted from the ventricular tissue samples ($n = 8$/group) using mirVana™ miRNA isolation kit (Ambion Inc., Austin, TX, USA). Quantitative PCR was performed using a LightCycler® 480 Real-Time PCR 96-well System II (Roche Diagnostic Corp., Indianapolis, IN, USA). The primers used were purchased from Applied Biosystems (Foster City, CA, USA), including: CSE (item# Mm00461247_m1), CBS (item# Mm00460654_m1), Prx5 (item# Mm00465365_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (item# Mm99999915_g1). Positive controls, negative controls, and in-run standard curve were used in each session of amplification. The expression level of mRNA for each of the targeted proteins (CSE, CBS, and Prx5) was normalized as the ratio to the housekeeping control gene GAPDH.

Western immunoblotting

The heart tissue samples ($n = 8$/group) were homogenized in ice-cold CelLytic™ MT mammalian tissue lysis/extraction reagent along with a protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA). The homogenate was centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was recovered and was as the total cellular protein. Seventy micrograms of total protein from each sample was separated by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE) on 10% (for CSE) or 18% (for Prx5) acrylamide gels and transferred to a nitrocellulose membrane, and then blocked with 5% nonfat dry milk in Tris Buffered Saline with Tween® (TBST). The membrane was subsequently incubated with the primary antibodies specific to CSE, CBS, Prx5 (item# 12217-1-AP, 14787-1-AP, and 17724-1-AP, Proteintech Group, Inc., Chicago, IL, USA), or VEGFR2 (item# 9698S, Cell Signaling Technology, Danvers, MA, USA) and subsequently the secondary antibody. The membranes were developed using enhanced chemiluminescence and exposed to X-ray film. For verifying equal sample loading, the membranes were reprobed with the primary antibody of actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a housekeeping control. All the Western blot bands were scanned for densitometry quantification and analysis with the BIOQUANT imaging software. The protein expression levels for each of the targeted proteins (CSE, CBS, Prx5, and VEGFR2) were normalized as the ratio to actin.

Statistical analysis

All measurements are expressed as group means ± standard error (SE). The data were analyzed by either unpaired *t*-test (between two groups) or one-way analysis of variance (ANOVA) (among >3 groups). If a significant value of *F* was obtained in ANOVA, the Student–Newman–Keuls post hoc test was further used for pair-wise comparisons. $P < .05$ was considered statistically significant.

Results

BRJ-induced infarct size reduction and its abolishment by PAG

As indicated by the representative images of TTC-stained heart slices (Figure 2(a)), myocardial infarct size following I/R injury was significantly smaller in the BRJ-treated mice ($15.8 \pm 3.2\%$ of risk area) as compared with the control group ($46.5 \pm 3.5\%$; $P < .05$, Figure 2(b)). The infarct-sparing effect of BRJ was abolished with PAG as shown by an increase in infarct size to $48.0 \pm 5.2\%$ ($P < .05$ vs. BRJ group). Animals treated with PAG alone did not alter infarct size ($48.8 \pm 2.7\%$), which was similar to the control group. In addition, the areas at risk (percentage of LV) were not significantly different among the four experimental groups ($P > .05$).

BRJ protects against left ventricular remodeling and dysfunction

None of the groups had significant LV dilatation at 24-h post infarction (Figure 3(c)), however, BRJ decreased LVESD (2.5 ± 0.1 mm) and preserved FS: $31 \pm 3\%$ as compared to control (LVESD: 3.3 ± 0.3 mm and FS: $15 \pm 3\%$, respectively; $P < .05$; Figure 4(c,d)). Baseline LVESD and FS were 2.0 ± 0.1 mm and $44 \pm 2\%$, respectively. Moreover, LV anterior wall diastolic thickness (LVAWDT) was preserved with BRJ (0.99 ± 0.06 mm) as compared to untreated mice (0.75 ± 0.04 mm, $P < .05$), indicating less anterior wall thinning with BRJ treatment following myocardial infarction (by LAD occlusion) and reflective of cardioprotection (Figure 4(f)). Baseline LVAWDT was 1.07 ± 0.04 mm, $P > .05$ versus BRJ. LVPWDT was not different between baseline (1.20 ± 0.03 mm), untreated controls (1.11 ± 0.06 mm) and BRJ-treated mice (1.14 ± 0.06 mm, $P > .05$, Figure 4(g)).

BRJ elevates H_2S levels in heart tissues

In the cardiac tissue samples collected from the BRJ-treated mice, H_2S levels were significantly enhanced (1.64 ± 0.03 μM/mg protein, $P < .01$) as compared to those from the control mice (0.87 ± 0.06 μM/mg protein, Figure 4).

Effects of BRJ on plasma levels of nitrate, nitrite, and NO_x

As shown in Figure 5(a,c), both nitrate and NO_x levels were significantly lower as compared with the Sham group ($P < .05$) in the plasma samples collected at the end of I/R. Plasma nitrite level remained unchanged (Figure 5(b)). Oral ingestion of BRJ did not restore the I/R-depressed plasma nitrate and NO_x levels ($P > .05$,

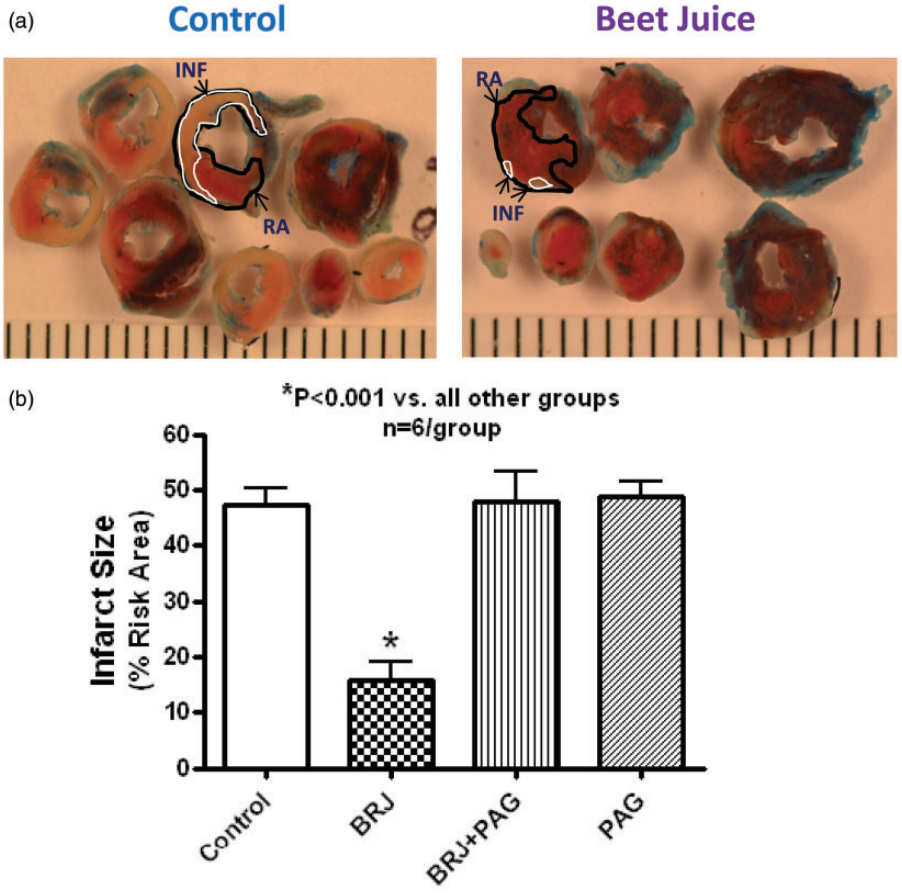

Figure 2 (a) Representative images of the TTC-stained heart sections from the mice treated with or without oral ingestion of BRJ. (b) Averaged myocardial infarct size (percentage of risk area) measured 24-h post-MI in the various groups (mean ± *SE*, $n = 6$/group). RA: risk area; INF: infarct zone. Note that infarct size was significantly reduced with BRJ, which was blocked by PAG (i.e. DL-propargylglycine – a specific inhibitor of H_2S-producing enzyme – cystathionine-γ-lyase)

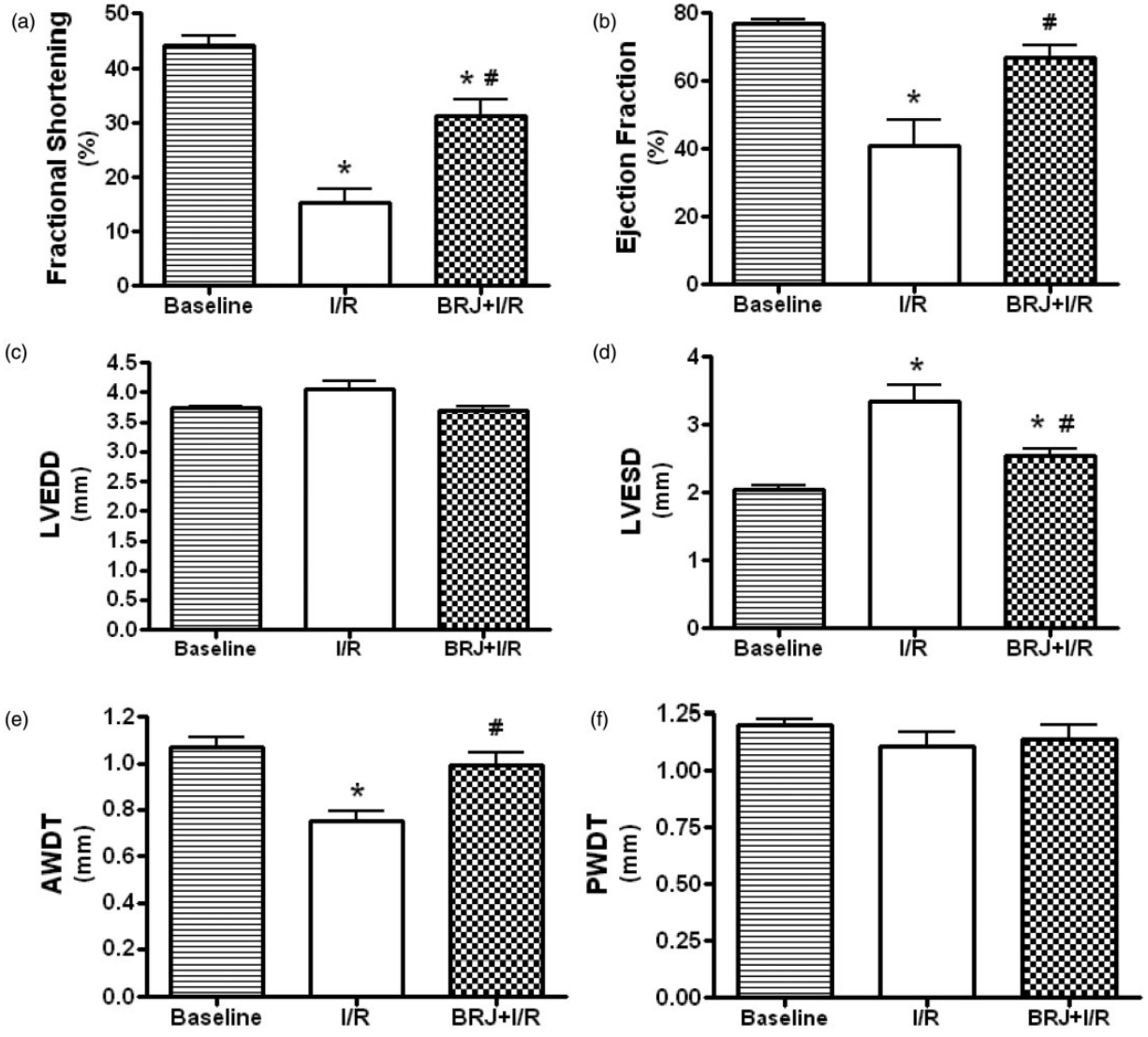

Figure 3 Preservation of postischemic LV contractility by oral ingestion of BRJ. The following echocardiography parameters in the control I/R and BJR-treated BRJ+I/R mice are presented: (a) fractional shortening; (b) ejection fraction; (c) LV end-diastolic diameter (LVEDD); (d) LV end-systolic diameter (LVESD); (e) Anterior wall diastolic thickness (AWDT); and (f) Posterior wall diastolic thickness (PWDT). Data are mean ± *SE*. **P* < .05 versus the composite mean baseline values ($n = 12$); #*P* < .05 versus I/R group ($n = 6$)

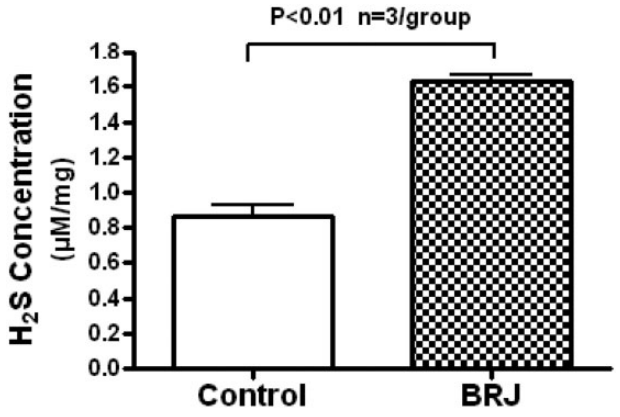

Figure 4 H_2S levels assessed in the heart tissues harvested seven days after BRJ ingestion, which show a significant increase in cardiac H_2S as compared with control hearts (mean ± *SE*, $n = 3$/group, $P < .01$). BRJ: beetroot juice

BJR + I/R group vs. I/R group, Figure 5(a,c)). Pretreatment of CSE inhibitor – PAG had no effect on the post-I/R nitrate or NO_x levels in plasma. BRJ intake alone did not cause any change in plasma nitrite concentration. However, PAG significantly potentiated the plasma nitrite levels ($P < .05$ vs. all other groups; Figure 5(b)), suggesting this drug may enhance nitrate-to-nitrite conversion and/or impede nitrite reduction.

BRJ does not alter cardiac expression of H_2S-producing enzymes

To investigate the effects of BRJ ingestion on cardiac mRNA and protein levels of two main H_2S-producing enzymes, CSE and CBS, we performed real-time PCR and Western blots in the heart tissue samples from the control and BRJ-fed mice ($n = 8$/group). No significant difference was found between the control and BRJ groups for either mRNA or

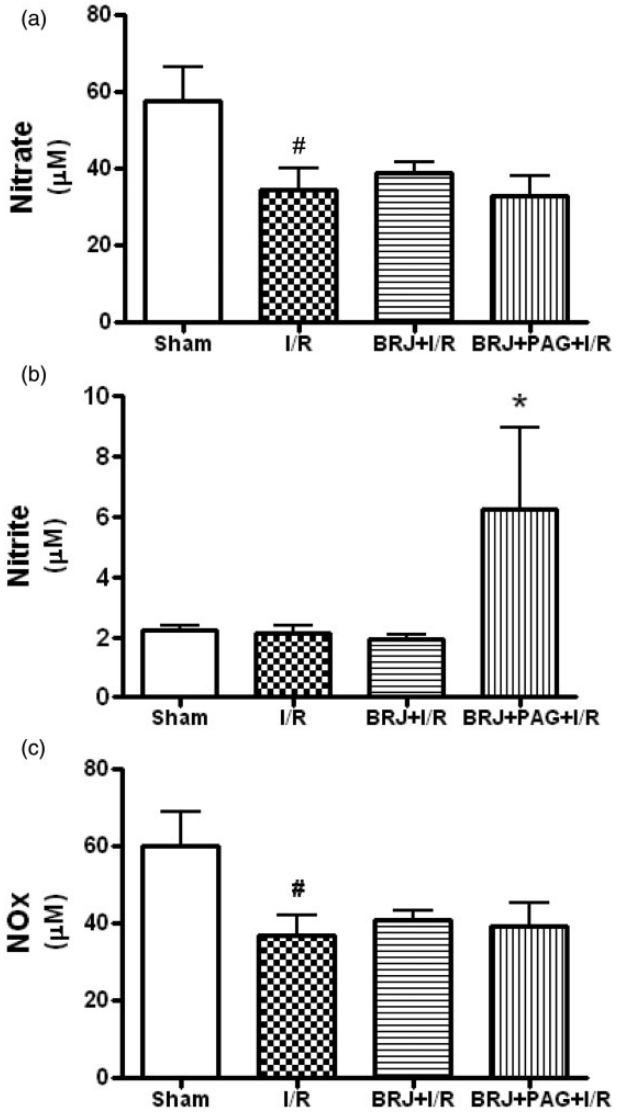

Figure 5 Assessment of plasma levels of nitrate (a), nitrite (b), and sum of nitrate and nitrite, i.e. NO_x (c) at the end of ischemia–reperfusion protocol. Data are mean ± SEM ($n = 4$–6/group). *Indicates $P < .05$ versus all other groups; #Indicates $P < .05$ versus Sham group. BRJ: beetroot juice; PAG: DL-propargylglycine

protein levels of CSE (Figure 6) or CBS (Figure 7). These results suggest that BRJ-induced H_2S production was not accompanied by *de novo* synthesis of CSE or CBS in heart tissues.

BRJ does not induce cardiac expression of Prx5

We further examined the effect of seven-day BRJ ingestion on cardiac expression of Prx5 – a mitochondria-localized antioxidant protein. The results show that BRJ treatment did not alter mRNA and protein levels of Prx5 in the mouse hearts (Figure 8, $n = 8$/group, $P > .05$ vs. control group).

BRJ increases cardiac expression of VEGFR2

Effects of seven-day BRJ ingestion on protein levels of vascular endothelial growth factor receptor 2 (VEGFR2) in the mouse hearts ($n = 8$/group) were determined with Western immunoblotting (Figure 9(a,b)). Significant difference ($P < .05$) was found between the control and BRJ groups.

Discussion

Red beetroot has not only the highest level of nitrate among vegetables[29] but also contains numerous bioactive agents, such as betaine, betanin, betaxanthins, flavonoids, polyphenols, vitamins (thiamine, riboflavine, pyridoxine, ascorbic acid), folic acid, and different metal elements.[23] In particular, betanin, flavonoids (e.g. quercetin), vitamin C (ascorbic acid), and other polyphenols have extremely effective antioxidant properties.[30] It has been increasingly appreciated that drinking BRJ promotes healthy benefits to cardiovascular system. Clinical studies in hypertensive patients showed that chronic ingestion of BRJ significantly lowered blood pressure.[31] The results from our present study have shown that the mice treated with BRJ for seven days prior to I/R had a remarkable 64% reduction in infarct size following I/R (Figure 2). Moreover, the BRJ-fed mice had significantly improved cardiac function as shown by decreased LVESD (2.5 ± 0.1 mm) and preserved FS: 31 ± 3% as compared to controls (Figure 3). We further demonstrated that the BRJ-induced cardioprotection was accompanied with significantly increased H_2S generation (Figure 4). Taken together, the present study has provided the first direct evidence that oral ingestion of BRJ is capable of reducing myocardial infarct size and improving post-I/R ventricular contractile function in a well-established mouse model of *in vivo* cardiac I/R injury.

H_2S is a potent gasotransmitter that is produced enzymatically on a continuous basis at micromolar levels in mammals and exerts a number of physiological actions in the cardiovascular system.[32] The production of H_2S in mammalian systems has been attributed to three key enzymes, CSE, CBS, and 3-mercaptopyruvate sulfurtransferase. CSE is believed to be the predominant H_2S-producing enzyme in the heart since it has been shown that genetic deletion of this enzyme in mice markedly reduces H_2S levels in the serum, heart, aorta, and other tissues and these animals display pronounced hypertension and diminished endothelium-dependent vasorelaxation.[33] Administration of the H_2S donor, sodium hydrosulfide, has been shown to reduce infarct size following I/R. Our previous work showed that tadalafil-induced cardioprotection against I/R injury through CSE-driven generation of H_2S and the infarct-sparing effect of tadalafil was abolished with the CSE inhibitor, PAG, as well as in CSE-knockout mice.[22] Additionally, the importance of CSE-driven H_2S in regulating blood pressure[34] may further explain the antihypertensive effects of many pharmacological or natural compounds, including BRJ. In the present study, CSE inhibition with PAG abolished the cardioprotective of BRJ as shown by an increase in infarct size as compared to BRJ-treated mice (Figure 2(a)). Moreover, myocardial H_2S levels were increased following oral ingestion of BRJ for seven days as compared to the untreated control hearts (Figure 4(a)). These results clearly suggest a causative role of CSE-generated H_2S in cardioprotection with BRJ.

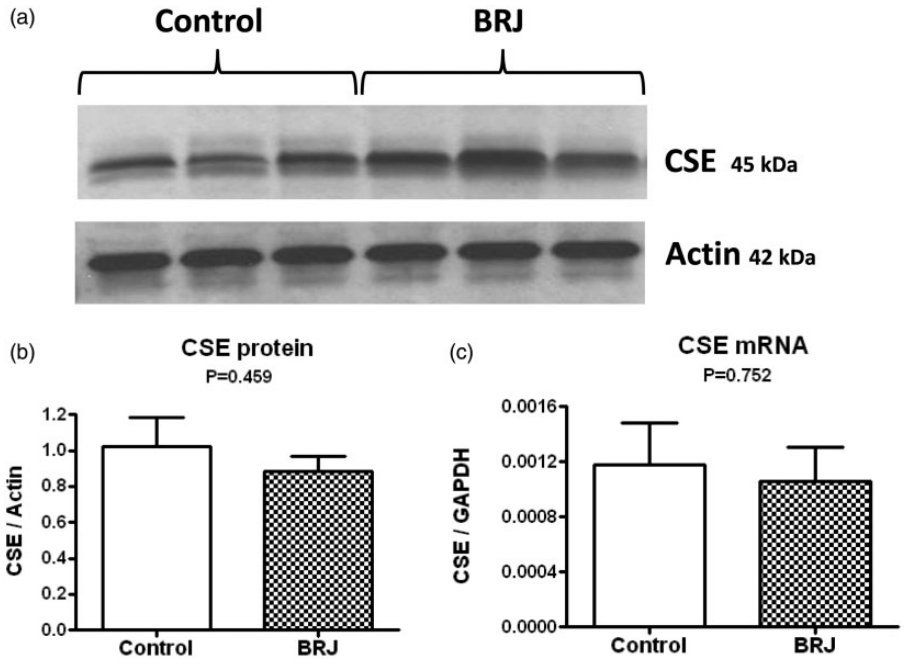

Figure 6 Effects of BRJ ingestion on cardiac mRNA and protein levels of CSE ($n = 8$/group). Western immunoblotting with densitometry analysis was used to quantify CSE protein expression (a and b) and real-time PCR was used for determining CSE mRNA levels (c). No significant difference was found in either of the parameters between the control and BRJ groups. BRJ: beetroot juice; CSE: cystathionine-γ-lyase

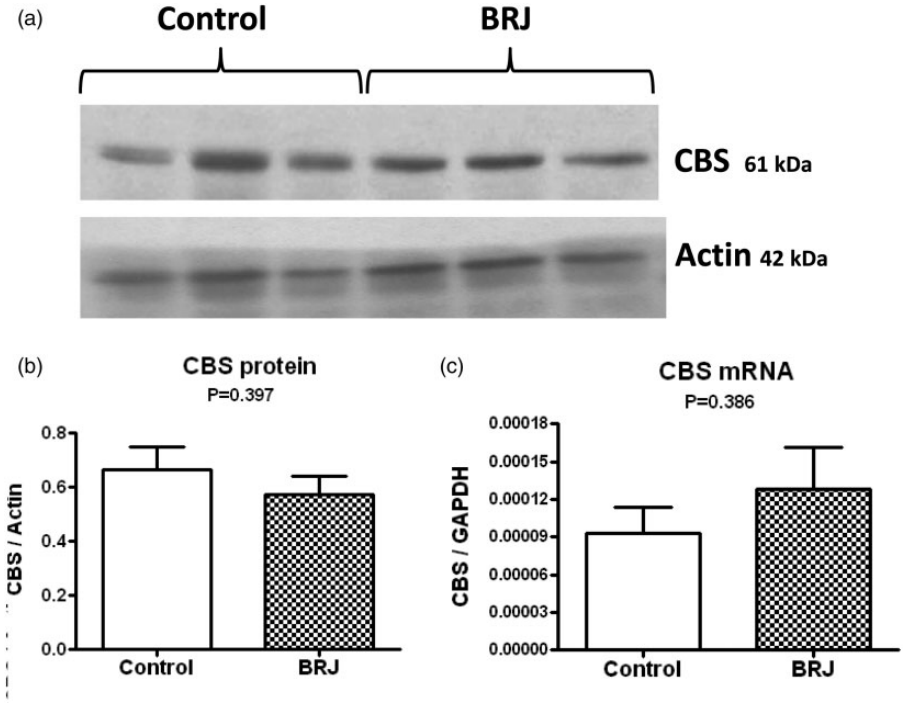

Figure 7 Effects of BRJ ingestion on cardiac mRNA and protein levels of cystathionine β-synthase (CBS), another major H_2S-producing enzymes ($n = 8$/group). Western immunoblotting with densitometry analysis was used to quantify CBS protein expression (a and b) and real-time PCR was used for determining CBS mRNA levels (c). No significant difference was found in either of the parameters between the control and BRJ groups. BRJ: beetroot juice

Our results also demonstrate preservation of LV function with BRJ treatment following myocardial infarction as compared to untreated controls (Figure 3). Although none of the groups presented with LV dilatation at 24 h post MI, BRJ attenuated the increase in LVESD seen in the control mice (Figure 3(d)) and preserved FS (Figure 3(a)). Moreover, the AWDT was also preserved in BRJ-treated mice as compared to controls (Figure 3(e)), indicating less anterior wall

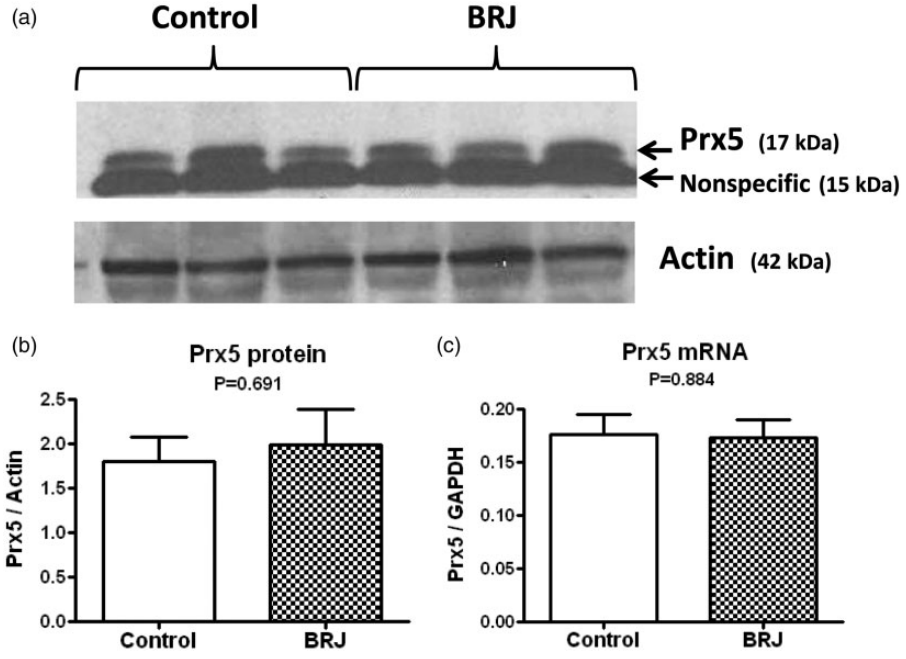

Figure 8 Effects of seven-day BRJ ingestion on mRNA and protein levels of peroxiredoxin 5 (Prx5) in the mouse hearts ($n = 8$/group). Western immunoblotting was used to detect Prx5 protein expression (a and b) and quantitative PCR was used for determining Prx5 mRNA levels (c). No significant difference was found in either of the parameters between the control and BRJ groups. BRJ: beetroot juice

thinning and less cardiomyocyte loss, which parallels our infarct size data. As expected, PWDT was not different between the groups, which confirmed our model of regional I/R involves only the anterior wall of LV following occlusion of the left anterior descending coronary artery.

Previous studies have shown that inorganic nitrates play an important role in protection of the heart against I/R injury. Supplemental nitrate in the drinking water for seven days increased blood and tissue NO products and significantly reduced infarct size.[10] We recently demonstrated that nitrate supplementation also attenuated cardiotoxicity of doxorubicin – a highly effective anticancer drug[13,14] and the protective effects of nitrate were associated with increase of plasma nitrate–nitrite levels[13] and upregulation of cardiac Prx5.[24] Contrary to our expectation, the present results indicated that the BJR-induced cardioprotection was not associated with significant restoration of plasma concentrations of nitrate and NO_x, which were significantly suppressed at the end of I/R (Figure 5). A plausible explanation for this discrepancy is the largely different doses of oral nitrate intake between the previous study using 10 g/L sodium nitrate (yielded 12-mM nitrate concentration)[10] and our current BRJ study (0.7-mM nitrate). It is apparent that BRJ ingestion is able to reduce myocardial infarct size without elevation of blood nitrate–nitrite levels (Figure 5). Interestingly, the plasma nitrite level was significantly elevated in the PAG + BRJ group where the BRJ-induced cardioprotection was blocked despite the high level of nitrite (Figure 5(b)). Nevertheless, we cannot completely rule out the participation of the nitrate–nitrite–NO conversion pathway under ischemic conditions as previously proposed.[10] The potential role of H_2S in BRJ-induced protection provides new insights into how BRJ may offer cardiovascular benefits. We do not know how PAG – an inhibitor of H_2S-producing enzyme potentiates nitrite conversion–production while abolishing the cardioprotective effects of BRJ. Further studies are needed to elucidate the possible interaction between H_2S and NO in the cardioprotective mechanisms of BRJ.

In the present study, the seven-day BRJ ingestion regimen did not alter mRNA or protein levels of Prx5 in the CD-1 mouse hearts (Figure 8). These results differ from our previous observations showing nitrate-induced increase of Prx5 expression in the heart, which was significantly reduced by doxorubicin in CF-1 mice.[13,24] Considering the absence of increased plasma nitrate–nitrite levels in the BRJ-treated CD-1 mice (Figure 5), it seems that the induction of cardiac Prx5 is associated with elevated NO levels rather than H_2S. Therefore, it appears that direct upregulation of this antioxidant enzyme may not play a major role in BRJ-induced cardioprotection against I/R injury. Nevertheless, the activity of Prx5, which is a part of the thioredoxin (Trx) system that ultimately controls the redox status, was not measured in our present study. In particular, as recently demonstrated by Stanley et al.,[35] we should consider the redox status of Trx2 that tightly control H_2O_2 emission from mitochondria and its close dependence on the levels of mitochondrial energetics. There is a possibility that H_2S may have an impact on enzymatic activities that rescues the redox status of Trx2 and its dependent Prx5. Hence, potential modulation of Trx2/Prx5 redox status by H_2S might play a role in BRJ-induced cardioprotection without increasing Prx5 protein levels. It is also noteworthy that other unidentified H_2S-dependent factors that could lend myocardium a favorable metabolic state in the BRJ-fed mice under the ischemic stress conditions. Particularly, it

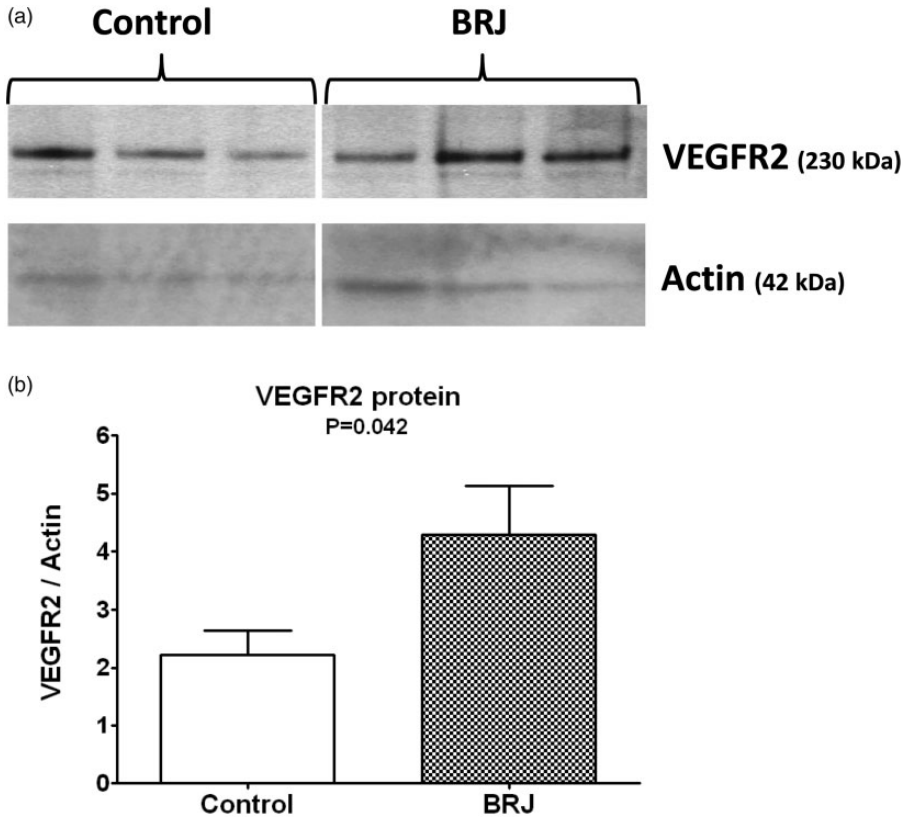

Figure 9 Effects of seven-day BRJ ingestion on protein levels of VEGFR2 in the mouse hearts ($n = 8$/group). Western immunoblotting was used to detect Prx5 protein expression (a and b). Significant difference ($P < .05$) was found between the control and BRJ groups. BRJ: beetroot juice; VEGFR2: vascular endothelial growth factor receptor 2

has been shown that BRJ intake reduced oxygen cost and enhanced skeletal muscular work efficiency as well as exercise performance in human subjects.[36–38] Whether such metabolic benefits are induced in the cardiac muscles of the BRJ-treated animals requires further investigation.

Furthermore, our observation on the enhancement of cardiac expression of VEGFR2 following seven days of BRJ intake (Figure 9) also suggests the involvement of H_2S signaling, because VEGFR2 was recently identified as a direct protein target of H_2S.[26,27] The exact mechanism in explaining what role is played by H_2S–VEGFR2 signaling network in the BRJ-induced cardioprotection require future in-depth studies. It is notable that VEGFR2 was recently shown to be downregulated in myocardium by I/R injury[39] or in hindlimb muscles by acute ischemia[40,41] in rodents. It is generally recognized that most biological functions of VEGF are mediated via VEGFR2, one of the two receptor protein tyrosine kinases, VEGFR1 (Flt-1) and VEGFR2 (KDR), to which VEGF binds.[42] Most notably, VEGF activates VEGFR2 and its downstream signaling targets involving activation of protein kinases (e.g. phosphoinositide 3-kinase (PI3K)/Akt, protein kinase C (PKC), extracellular signal-regulated kinase (ERK)), and endothelial NO synthase (eNOS)-mediated NO production, which are known to be cytoprotective.[42] VEGFR2 was previously demonstrated to mediate the neuronal or vascular protection by hypoxia preconditioning in neonatal brain[43,44] and by low-energy extracorporeal shock wave in hindlimb muscles.[41] However, to our best knowledge, the role of VEGFR2 has not been well defined in myocardial protection. Therefore, our initial evidence on a potential involvement of the upregulated VEGFR2 in BRJ-induced cardioprotection (Figure 9) is intriguing and apparently warrants future studies in order to gain a better understanding in VEGFR2-mediated cardioprotective signaling cascades and their interactions with NO and H_2S in general following nutraceutical intake of BRJ.

Nevertheless, our current study has several limitations. For instance, we did not measure the serum and/or cardiac levels of nitroso products (RXNO), which are frequently increased in tissues after nitrate intake even in the absence of a detectable nitrite increase. The confounding factors that may influence the plasma measurements of nitrate–nitrite include the variable amount of water–food intake during the 24 h of reperfusion period before the mice were sacrificed for blood sampling (for measuring nitrate/nitrite) and heart tissue harvesting (for determining infarct size). Therefore, the present study cannot rule out the participation of nitrate–nitrite–NO signaling pathway in the BRJ-induced cardioprotection against I/R injury. It is noticeable that a previous study employed a comparable low dose of sodium nitrate ingestion (i.e. 0.1 mmol/kg/day) in rats, which led to significant cardiorenal protective effects against the pathological changes caused by salt-induced hypertension.[45] There is a high probability that such a low dose of sodium nitrate may protect against I/R injury as well. In addition, the BRJ products used our current study had apparently a moderate concentration of nitrate

(0.7 mM), as compared with other BRJ brands such as Beet It® (containing 11 mM nitrate) that were previously used in several human studies. Hence, it is plausible that other NO-related mechanisms could be also implicated when the BRJ products with higher nitrate concentrations are applied.

Future investigations should also address a number of unsolved questions on the H_2S-dependent molecular mechanisms of BRJ-induced cardioprotection. For instance, it remains to be confirmed if the increased H_2S levels after BRJ ingestion may restore cardiac redox balance interacting with oxidized thiols via the formation of persulfides as recently suggested by Francoleon et al.[46] The role of protein sulfhydration in BRJ-induced cardioprotection should be elucidated in the future. Another limitation of the current study is that we did not measure CSE enzyme activity, which could be important under the observed absence of CSE mRNA and protein induction by BRJ. It should be emphasized that not only H_2S production but also H_2S mobilization from existing reservoirs are equally important for the action of this signal transmitter. Also it would be interesting to delineate the possible interaction between H_2S and NO in the *in vivo* model of cardioprotection, through either direct chemical interaction[47] or indirectly via modulation of NO- or H_2S-producing enzymes.[48] Finally, our group recently showed that administration of an H_2S donor (Na_2S) during I/R in mice prevents the formation and the activation of the cryopyrin inflammasome, a macromolecular complex responsible for sensing tissue injury, amplifying the inflammatory response, and inducing cell death.[49] It remains to be determined if a natural botanical product as BRJ has a similar anti-inflammatory potency to those of the chemical H_2S donor in the setting of cardiac I/R injury.

In conclusion, our results provide direct evidence that oral ingestion of BRJ reduces myocardial infarction and LV contractile dysfunction following I/R injury by increasing H_2S generation in the heart tissues. These beneficial effects of BRJ appear to be independent of increased nitrate–nitrite levels or induction of cardiac antioxidant enzymes. We believe that these studies may have potential translational significance because dietary intake of BRJ could be a natural, safe, and inexpensive nutraceutical strategy to prevent and/or alleviate myocardial I/R injury. In support of this notion, a recent study in human volunteers has demonstrated significant blood pressure lowering effect of BRJ and novel varieties of beetroot-enriched breads.[50] Based on the current study, we propose that further clinical studies are needed in the patients with acute myocardial infarction or undergoing cardiac bypass surgery to demonstrate the effect of BRJ in reducing infarct size and improvement of postischemic cardiac function recovery.

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