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Beetroot and sodium nitrate ameliorate cardiometabolic changes in diet-induced obese hypertensive rats

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Abstract

Scope: Dietary intake of beetroot by humans reduces blood pressure but whether this is caused by nitrate or betanin is not well-defined; neither are effects on other signs of metabolic syndrome. **Methods and results:** Rats fed a high-carbohydrate, high-fat diet (H) for 16 weeks developed abdominal obesity, hypertension, altered cardiovascular and liver structure and function, and impaired glucose tolerance compared to rats fed a corn starch diet (C). H rats treated with ~16 mg/kg/day of nitrate either from beetroot juice (H+B) or sodium nitrate (H+N) for the last 8 weeks reduced systolic blood pressure by ~25 mmHg, improved cardiac structure and function, plasma lipid profile and plasma markers of liver function, reduced inflammatory cell infiltration in heart and liver and decreased left ventricular fibrosis. In the left ventricle, H rats increased mRNA expression of

connective tissue growth factor (CTGF), monocyte chemoattractant protein 1 (MCP-1), matrix metalloproteinase-2 (MMP-2) and adenosine monophosphate-activated protein kinase-alpha (AMPK-α) and decreased mRNA expression of peroxisome proliferator-activated receptor-alpha (PPAR-α); both beetroot and sodium nitrate diet-fed rats decreased CTGF three-fold, MCP-1 and MMP-2 two-fold, and doubled PPAR-α mRNA expression in left ventricular tissue.
Conclusion: The similar functional and molecular responses to beetroot and sodium nitrate indicate that the nitrate content of beetroot reduced inflammation and improved cardiovascular, liver, and metabolic function in rats with metabolic syndrome, rather than betanin.

Key words: Beetroot; Cardiovascular complications; Metabolic syndrome; Sodium nitrate

Abbreviations: C, corn-starch rich diet; H, high-carbohydrate, high-fat diet; B, beetroot; N, sodium nitrate; NO, Nitric oxide; CTGF, connective tissue growth factor; MMP-2, matrix metalloproteinase-2; MCP-1, monocyte chemoattractant protein-1; TGF-β, transforming growth factor beta; AMPK-α, adenosine monophosphate-activated protein kinase alpha; PPAR-α, Peroxisome proliferator-activated receptor alpha; OGTT-AUC, oral glucose tolerance test-area under the curve; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; NEFA, non-esterified fatty acids; LDL, low-density lipoprotein; IVSd, interventricular septal end diastole; LVIDd, left ventricular internal diameter end diastole; LVPWd, left ventricular posterior wall end diastole; IVSs, interventricular septal end systole; LVIDs, left ventricular internal diameter end systole; LVPWd, left ventricular posterior wall end systole; dP/dt, left ventricular contractility.

1 Introduction

Obesity is associated with an increased risk of hypertension, coronary heart disease, and myocardial infarction leading to heart failure [1, 2]. In recent years, many national health authorities have focused on improvement of diet, particularly in population groups with an increased risk of cardiovascular disease [3-5]. Many organisations including the World Health Organization are aiming to decrease the prevalence of dietary-related diseases through evidence-based recommendations on nutrition and food consumption. This includes promotion of a healthy diet, including the consumption of five or six servings of vegetables and two servings of fruits per day [6, 7], although only 8.9% of US adults met vegetable intake recommendations in 2013 [8]. A lower intake of fruits and vegetables is associated with a higher risk of mortality [9-12]. The Mediterranean diet is one widely-studied example where increased intake of fish, monounsaturated fats from olive oil, fruits, vegetables, whole grains, legumes/nuts, and moderate alcohol consumption reduced the incidence of cardiovascular disease, diabetes, and obesity [13]. There is considerable evidence that an increased intake of vegetables and fruits decreased the risk of hypertension, coronary heart disease, and stroke [14] and increased intake of fruit and leafy green vegetables reduced the risk of type 2 diabetes [15]. Non-alcoholic fatty liver disease [16] and hypertension [17], both associated with obesity, were prevented or improved by an increased intake of fruits or vegetables.

These diet-induced changes could be caused by many bioactive compounds in foods [18, 19], of which nitrate is one. Increased consumption of dietary fruits and vegetables containing nitrate reduced blood pressure [20], but the mechanisms for these effects are unclear [21].

Increased dietary intakes of nitrate-rich vegetables reduce the risk of cardiovascular disease [22]. Unlike red-purple fruits such as berries and plums which contain anthocyanins, red beetroot contains betanin, a nitrogen-containing water-soluble betacyanin from the larger group of betalains, as well as inorganic nitrate. Both red and white beetroot-enriched bread decreased blood pressure [23] suggesting that betanin is not required to reduce blood pressure. However, betanin may have other beneficial effects, possibly reducing total cholesterol, preventing LDL oxidation and protecting from DNA damage [24, 25].

Inorganic nitrate (NO_3^-) in the diet is reduced to nitrite (NO_2^-), and then to nitric oxide (NO) by salivary bacteria and the acidic environment of the stomach [20, 26]. Endogenous NO produced by NO synthase in endothelial cells mediates vascular dilation and inhibits infiltration of inflammatory cells and platelet aggregation [27]. Increased consumption of inorganic nitrate in green leafy vegetables and beetroot have potential cardiovascular benefits [28, 29]. Recently, a meta-analysis and several clinical studies [30-33] have suggested that dietary inorganic nitrate or beetroot juice reduced plasma LDL, and systolic blood pressure, with improved endothelial and cardiac function [26, 34-37].

However, there is no clear evidence that increased dietary betanin or inorganic nitrate protect against cardiovascular and liver damage in metabolic syndrome. Thus, we have studied beetroot juice as a source of nitrate for attenuation of organ structure and function in rats fed a high-carbohydrate, high-fat diet as a model of metabolic syndrome and compared responses with a similar dosage of nitrate from sodium nitrate. Cardiovascular, liver, and metabolic parameters were measured during both beetroot and sodium nitrate treatment along with heart, liver, and skeletal muscle gene expression to identify possible mechanisms mediating the changes to these organs.

2 Materials and methods

2.1. Rats and diets

72 male Wistar rats (8-9 weeks old) were randomly divided into 6 experimental groups (n=12 each, with two rats from each group randomly chosen for histological study) and fed either corn starch diet (C), C + sodium nitrate (C+N), C + beetroot juice (C+B), high-carbohydrate, high-fat (H), H + sodium nitrate (H+N) or H + beetroot juice (H+B) for a total of 16 weeks. The composition of C and H diets are detailed in Supplementary table 1. Beetroot juice (commercially available as Beet It shots, James White, UK) from a single batch was purchased from a local health-food shop and sodium nitrate was purchased from Sigma-Aldrich Australia (Sydney, NSW, Australia). Studies using Beet It doses of 70 ml report that each dose contains ~400 mg nitrate [38], ~21 mg betanin, ~1.02 mg betaxanthin, and ~22.6 mg total flavonoids (rutin equivalent) [39]. C, C+B, and C+N rats were fed with C diet for the first 8 weeks and then with C, C + B or C + N diets for the last 8 weeks. H, H+B, and H+N rats were fed with H diet for the first 8 weeks and then with H, H + B, or H + N diets for the last 8 weeks. Beetroot juice 50 ml/kg food (containing ~280 mg nitrate) and sodium nitrate 400 mg (containing ~290 mg nitrate) dissolved in 5 ml of water added as 5 ml/kg food were supplemented by replacing equivalent volumes of water from the diets to achieve a dose of ~16mg/kg/day of nitrate based on daily food intake measurements. All experimental protocols were approved by the Animal Ethics Committee of the University of Southern Queensland (13REA005 valid from September 2013 to September 2015). At the end of 16 weeks, measurements of body composition,

oral glucose tolerance, plasma biochemistry, and histology were performed [40]; protocols are detailed in Supplementary Methods.

2.2. Cardiovascular measurements.

Systolic blood pressure was measured at 0, 8, and 16 weeks under light sedation following intraperitoneal injection with Zoletil (toletamine 10 mg/kg, zolazepam 10 mg/kg; Virbac, Peakhurst, NSW, Australia) [40-42]. Measurements were performed using an MLT1010 Piezo-Electric Pulse Transducer (ADInstruments, Bella Vista, NSW, Australia) and an inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer and PowerLab data acquisition unit (ADInstruments).

Echocardiographic examinations using a Hewlett Packard Sonos 5500 12 MHz transducer were performed to assess the cardiovascular structure and function at 16 weeks under anesthesia with intraperitoneal Zoletil and Ilium Xylazil (xylazine 6 mg/kg, IP; Troy Laboratories, Smithfield, NSW, Australia), in accordance with the guidelines of the American Society of Echocardiography using the leading-edge method [40-42].

Terminal anesthesia was induced via intraperitoneal injection of pentobarbitone sodium (Lethabarb, 100 mg/kg, Virbac, Peakhurst, NSW, Australia). After heparin (Sigma-Aldrich Australia) administration (200 IU) through the right femoral vein, a blood sample (~6 ml) was then collected from the abdominal aorta into heparinized tubes. Immediately after terminal anesthesia, left ventricular function was assessed using the isolated Langendorff heart preparation [40-42]. Isovolumetric ventricular function was measured by inserting a latex balloon catheter into the left ventricle of the isolated heart connected to a Capto SP844 MLT844 physiological pressure transducer and Chart software on a MacLab system (ADInstruments).

Thoracic aortic rings (~4 mm in length), collected <5 minutes after terminal anesthesia, were suspended in an organ bath chamber filled with Tyrode physiological salt solution bubbled with 95% O_2-5% CO_2 and allowed to stabilize at a resting tension of 10 mN. Cumulative concentration-response curves (contraction) were obtained for noradrenaline (Sigma-Aldrich Australia), and cumulative concentration-response curves (relaxation) were obtained for sodium nitroprusside or acetylcholine (Sigma-Aldrich Australia) following submaximal (~70%) contraction to noradrenaline [40-42].

2.3. Real-time polymerase chain reaction

Immediately following euthanasia, left ventricle, liver, and skeletal muscle portions were snap-frozen in liquid nitrogen and stored at −80°C in 5-ml cryovial tubes until quantitative analysis via real-time PCR. Total RNA was extracted from approximately 15 mg of tissue using 1000 mg of ceramic/silica beads in TRIzol® Reagent (Invitrogen, Melbourne, Australia) [43]. Extracted RNA concentration was quantified spectrophotometrically at 260 nm and DNase treated using the RQ1 RNase-free DNase kit (Promega Corporations, Madison, USA) to ensure the sample was free from DNA contaminants. First strand cDNA was then generated from 0.3 µg of template RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, USA) using random hexamers and oligo dTs [43]. cDNA was stored at -20 °C for subsequent analysis.

Real-time PCR was conducted using MyiQ™ single color 'real-time' PCR detection system (Bio-Rad Laboratories, Hercules, CA) with iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, USA) as the fluorescent agent. Forward and reverse oligonucleotide primers for the genes of interest were

designed using OligoPerfect™ Suite (Invitrogen, Melbourne, Australia) (Supplementary table 2). To compensate for variations in RNA input amounts and reverse transcriptase efficiency, mRNA abundance of the genes of interest was normalized to the housekeeping gene, β-actin, for heart, liver, and skeletal muscle. Real-time PCR reactions were run for 50 cycles of 95°C for 15 sec and 60°C for 60 sec. Relative changes in mRNA abundance were quantified using the $2^{-\Delta\Delta CT}$ method [44] and reported in arbitrary units. Ct values for β-actin were not altered by dietary intervention.

2.4. Statistical analysis

All data are presented as mean ± SEM. Results were tested for variance using Bartlett's test and variables that were not normally distributed were transformed (using log 10 function) prior to statistical analyses. Data from C, C+B, C+N, H, H+B, and H+N groups were tested by two-way ANOVA. When interaction and/or the main effects were significant, means were compared using Newman-Keuls multiple comparison *post hoc* test. Where transformations did not result in normality or constant variance, a Kruskal-Wallis non-parametric test was performed. A P-value of < 0.05 was defined as statistically significant. All statistical analyses were performed using GraphPad Prism version 6.00 for Windows (San Diego, CA, USA).

3 Results

3.1. Dietary intake, body composition and plasma biochemistry

Food, water, and energy intakes were unaltered in C+B and C+N rats compared to C rats and in H+B and H+N rats compared to H rats (Table 1, Supplementary Table 3). H rats increased feed conversion efficiency, body weight gain, abdominal circumference, and body mass index compared to C rats, while these parameters were reduced in H+B and H+N rats (Supplementary Table 3). H+B and H+N rats had unchanged total body fat and lean mass compared to H rats and increased total body fat and lean mass compared to C, C+B, and C+N rats (Table 1).

Plasma concentrations of total cholesterol, triglycerides, and non-esterified fatty acids (NEFA) were increased in H rats compared to C, H+B or H+N rats, while C rats had higher total cholesterol concentrations than H+B and H+N rats (Table 1). H rats also had higher fasting blood glucose concentrations compared to C rats; H+B and H+N rats showed decreased fasting blood glucose concentrations. Areas under the glucose concentration curve were greater in H rats than C rats (Table 1). H+B and H+N rats improved plasma glucose clearance compared to H rats with H+N rats showing greater plasma glucose clearance than H+B rats (Table 1). Plasma insulin concentrations almost doubled in H rats compared to C rats and insulin concentrations were reduced in H+B and H+N rats (Table 1).

3.2. Cardiovascular structure and function

H rats showed increased left ventricular internal diameter in diastole (LVIDd), relative wall thickness and left ventricular wet weight as signs of eccentric hypertrophy compared to C rats (Table 2). H rats showed impaired systolic function seen as decreased fractional shortening, developed pressure, and left ventricular contractility (dP/dt) with increased left ventricular diameter in systole (LVIDs), diastolic stiffness, and systolic wall stress (Table 2). H rats showed increased diastolic and systolic

stroke volumes, cardiac output, and estimated left ventricular mass compared to C rats, with increased systolic blood pressure and heart rate (Table 2).

Left ventricular diastolic and systolic diameters (LVIDd and LVIDs) were normalized in H+B and H+N rats compared to C rats and decreased compared to H rats, with no change in left ventricular posterior wall thickness in systole and diastole. These effects were accompanied by increased fractional shortening in H+B and H+N rats (Table 2). Diastolic stiffness, systolic volumes, cardiac output, systolic wall stress, and wet weight of left ventricle with septum were normalized and heart rate was decreased in H+B and H+N rats. Diastolic volumes and ejection times along with left-ventricular developed pressures were increased in H+B and H+N rats compared to C and H rats (Table 2). H+B and H+N rats showed normalized estimated left ventricular mass and wet weight as well as systolic blood pressure (Table 2).

Isolated thoracic aortic rings from H rats showed decreased vascular contraction with noradrenaline (Figure 1A) and decreased relaxation with sodium nitroprusside and acetylcholine compared to C rats (Figure 1B and C). H+B and H+N rats showed improved contraction and relaxation in isolated thoracic aortic rings (Figure 1A, B and C), which is similar to the changes in blood pressure.

C, C+B and C+N rats showed minimal infiltration of inflammatory cells (Figure 2A, B, and C) and interstitial collagen deposition (Figure 2G, H, I) with tissue morphology appearing normal. H rats showed increased infiltration of inflammatory cells (Figure 2D), with decreased inflammatory cells in H+B and H+N rats (Figure 2E, F). Interstitial collagen deposition was increased in H rats (Figure 2J) compared to C rats and decreased in H+B and H+N rats (Figure 2K, L) compared to H rats.

In the left ventricle, H rats increased expression of CTGF, MMP-2, MCP-1, and AMPK-α (Figure 3A, B, C, and E and Supplementary Table 4) and decreased PPAR-α mRNA (Figure 3F and Supplementary Table 4) compared to C rats. H rats showed no change in mRNA expression of transforming growth factor beta (TGF-β) in the left ventricle (Figure 3D and Supplementary Table 4) compared to C rats. In the left ventricle, H+B and H+N rats decreased CTGF, MMP-2, MCP-1, and AMPK-α mRNA expression (Figure 3A, B, C, and E and Supplementary Table 4) with increased PPAR-α mRNA expression similar to C rats (Figure 3F) and no change in TGF-β mRNA expression (Figure 3D and Supplementary Table 4) compared to H rats.

3.3. Liver structure and function and mRNA expression in liver and skeletal muscle tissue

Compared to C rats, H rats increased plasma activities of alanine transaminase (ALT), alkaline phosphatase (ALP), and aspartate transaminase (AST) as markers of liver damage; H+B and H+N rats decreased these parameters compared to H rats. Plasma activities were unchanged in C+B and C+N rats compared to C rats, except an increased plasma ALP activity in C+B and C+N rats compared to C rats (Table 2). The minimal liver lipid deposition and inflammatory cell infiltration in C rats (Figure 4A) was also shown in C+B and C+N rats and tissue morphology appeared normal (Figure 4B, C). Macrovesicular steatosis and portal inflammation was increased in H rats (Figure 4D) while H+B and H+N rats showed decreased inflammatory cell infiltration (Figure 4E, F) compared to H rats. No changes in AMPK-α or PPAR-α mRNA expression were observed in the liver (Figure 5A, B and Supplementary Table 4) or skeletal muscle (Figure 5C, D and Supplementary Table 4).

4 Discussion

Feeding rats with a high-carbohydrate, high-fat diet induces cardiovascular, liver, and metabolic changes including endothelial dysfunction [40]. Endothelial dysfunction with decreased production of NO leads to structural damage of the cardiovascular system and decreased function [40] resulting in hypertension [45], atherosclerosis [46], and stroke [47]. The current study used a dose of ~16 mg/kg/day nitrate in rats which is equivalent to a dose of nitrate of ~250-280 mg/day in 70-80 kg humans, based on body surface area comparisons between rats and humans [48]; this daily dose in humans is achieved with 50 ml of Beet It stamina shot. Similar responses with beetroot and sodium nitrate treatment suggest that the observed effects are due to nitrate present in beetroot, rather than betanin. Additionally, the dose of betanin in the present study is comparatively low to have clinical efficacy [49]. Further, betanin showed a bioavailability of up to 2.7% [24].

The H rats displayed most of the cardiometabolic signs that occur in humans with metabolic syndrome. We have shown that hypertension, left ventricular fibrosis with increased stiffness and vascular dysfunction occurs together with increased left ventricular CTGF and MMP-2 mRNA expression. Rats fed with H diet increased inflammatory cell infiltration in left ventricle which occurs together with increased MCP-1 mRNA expression [50]. We suggest that the decreased blood pressure and diastolic stiffness with both interventions is due to decreased collagen deposition in the left ventricle and normalized CTGF mRNA expression. Overexpression of MMP-2 and CTGF increased collagen deposition in heart and aortic walls [51, 52] and this relationship was also observed in H rats. CTGF activated by upstream TGF-β signaling stimulated the proliferation of connective tissue cells such as fibroblasts and extracellular matrix [53]. In the current study, decreased collagen deposition occurred with no change in expression of the upstream activator TGF-β mRNA, which is contradictory to previous studies [54].

Our results also suggested that beetroot and sodium nitrate interventions improved vascular function by improving endothelial-independent smooth muscle function, possibly by *in vivo* conversion of nitrate to nitrite, leading to increased vascular NO concentrations [20, 55]. NO modulated the release of cytokines and cell adhesion molecules to decrease inflammation [56], increase cyclic guanosine monophosphate (cGMP) in smooth muscle [57], and improve vascular function [58]. This improved vascular function is consistent with previous studies suggesting that NO normalized expression of MCP-1 mRNA. Additionally, NO inhibited the synthesis and expression of inflammatory cytokines such as TNF-α, induced signal transducer and activator of transcription (STAT3) phosphorylation, inducible nitric oxide synthase and cell adhesion molecules that attract inflammatory cells into the vessel wall [59]. These effects were mediated by inhibiting the activation of nuclear factor-κB which binds to the promoter regions of genes that code for pro-inflammatory proteins [60]. Extending these effects could be the mechanism for reduced inflammatory cell infiltration in the left ventricle and liver observed for both beetroot and sodium nitrate treatments.

Both beetroot and sodium nitrate treatment reduced plasma activity of liver enzymes and concentration of free fatty acids possibly leading to the decreases in liver fat vacuoles, inflammation, and liver weight. NO activates AMPK-α, an energy-sensing enzyme activated in response to cellular stress and by increased intracellular cGMP [61]. Beetroot juice increased basal oxidative metabolism and glucose uptake in C2C12 myocytes with elevated metabolic gene expression including peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α), nuclear respiratory

factor 1, mitochondrial transcription factor A, and glucose transporter 4, leading to increased mitochondrial biogenesis [62]. In our study, both beetroot and sodium nitrate interventions improved glucose tolerance independent of changes in body weight, similar to a previous study with sodium nitrate treatment in obese ZSF1 rats suggesting that nitrate regulated glucose uptake through SIRT3 (sirtuin 3)-AMPK activation in skeletal muscle [63]. Additionally, AMPK activation in liver was not altered with sodium nitrate treatment, suggesting AMPK-GLUT-4 mediated glucose uptake in skeletal muscles improved glucose homeostasis [63]. In contrast, in our diet-induced model, neither beetroot nor sodium nitrate treatment altered AMPK-α mRNA expression in skeletal muscle. In the heart, the increased AMPK-α expression in H diet rats was normalized in beetroot and nitrate-treated rats, suggesting that reduced blood pressure and improved cardiovascular function with either treatment reduced the cellular stress and workload, thereby normalizing AMPK-α expression.

The activation of PPAR-α during energy deprivation regulated lipid metabolism in the liver and increased NO production [64]. Few studies have explained how the improved relationship between PPAR-α and NO production benefits cardiovascular health [64]. Activation of PPAR-α also increased mitochondrial fatty acid β-oxidation [65]. Treatment with beetroot or sodium nitrate did not change PPAR-α mRNA expression in liver or skeletal muscle but increased expression in the heart. Similarly, low dose treatment with nitrate did not alter PPAR-α activation and expression in muscle and liver [66]. However, plasma triglycerides and NEFA were decreased as well as fat vacuoles in the liver in the current study. These results suggest that regulation of PPAR-α expression is tissue-specific during beetroot and nitrate treatment. Additionally, endothelial NO synthase-deficient mice treated with sodium nitrate decreased body weight and visceral adiposity [37], whereas in our rats with diet-induced metabolic syndrome, nitrate either as sodium nitrate or in beetroot juice had no effect on body weight and adiposity.

5 Conclusions

Our integrated study shows that, in a rat model of metabolic syndrome, supplementation with beetroot juice or sodium nitrate has similar effects in reducing blood pressure, plasma total cholesterol, triglycerides, and non-esterified fatty acids. Cardiovascular function and structure were also improved, along with normalization of CTGF, MCP-1, and MMP-2 mRNA expression in heart. These benefits were observed with a similar dose of nitrate from beetroot or sodium nitrate, suggesting that beetroot juice can be utilized as a safe and effective therapeutic intervention to provide nitrate to improve hypertension and cardiometabolic changes. Additionally, both interventions improved hepatic structure and glucose metabolism, and decreased plasma insulin and liver enzymes, without alterations in body weight, total fat, or expression of AMPK-α or PPAR-α mRNA. However, further investigation on beetroot and sodium nitrate will be necessary to understand the mechanisms underlying the improvements of the cellular changes seen in the metabolic syndrome.

6 Limitations

This study has measured responses at 0, 8 and 16 weeks although only parameters in living rats could be measured at 0 and 8 weeks. This assumes that all other parameters at 0 and 8 weeks before interventions were started are as previously reported by us [40]. Further, the initial onset of improvements with the interventions cannot be determined. Also, histopathology data is qualitative rather than quantitative.

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Author contributions

M.B., L.B and M.M. developed the original study aims and analyzed and interpreted the data; M.B. conducted the experiments. A.McA. assisted in gene expression experiments and data interpretation. M.B. and M.M. prepared manuscript drafts, with all authors contributing to the final version. M.M. has been the corresponding author throughout the writing process. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Cumulative concentration-response curves for noradrenaline (A), sodium nitroprusside (B) and acetylcholine (C) in thoracic aortic rings from C, C+B, C+N, H, H+B, and H+N rats. Data are shown as mean ± SEM. End-point means without a common letter in each data set significantly differ, P<0.05 and n=10/group. C, corn-starch rich diet; H, high-carbohydrate, high-fat diet; B, beetroot; N, sodium nitrate.

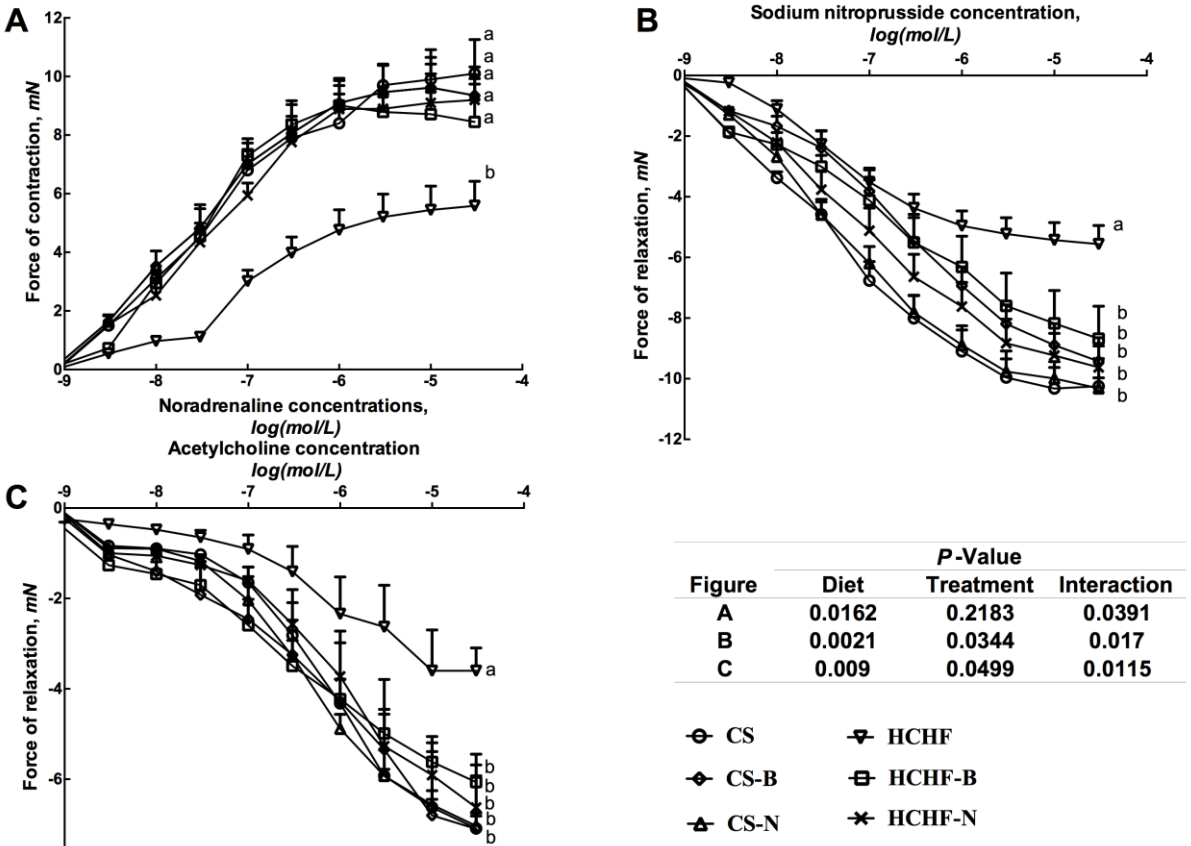


Figure	*P*-Value Diet	Treatment	Interaction
A	0.0162	0.2183	0.0391
B	0.0021	0.0344	0.017
C	0.009	0.0499	0.0115

Figure 2. Hematoxylin and eosin staining of left ventricle (original magnification ×20) showing inflammatory cells (marked as "in") as dark spots outside the myocytes in C (A), C+B (B), C+N (C), H (D), H+B (E), and H+N (F) rats. Picrosirius red staining of left ventricular interstitial collagen deposition (original magnification ×20) in C (G), C+B (H), C+N (I), H (J), H+B (K), and H+N (L) rats; collagen deposition is marked as "cd", and hypertrophied cardiomyocytes are marked as "hy".

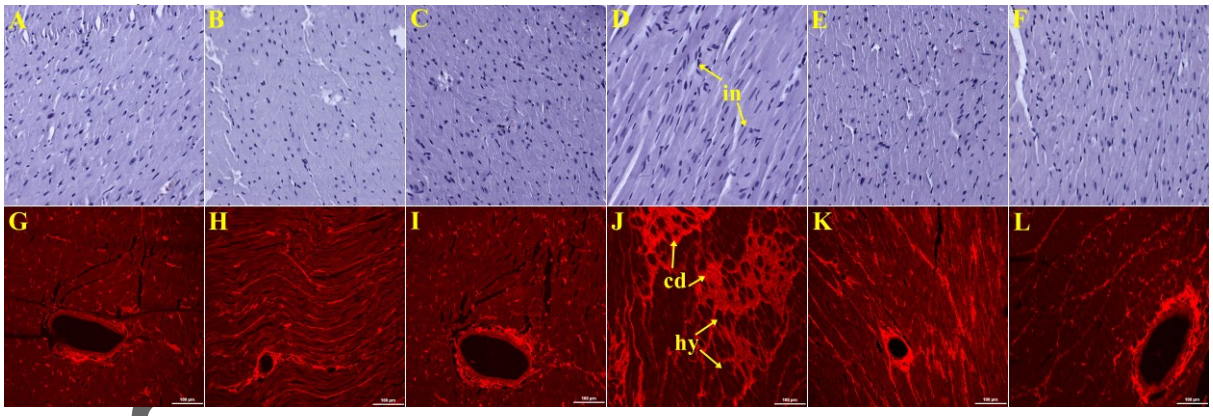


Figure 3. Left ventricular mRNA expression of CTGF (A), TGFβ (B), MCP1 (C), MMP2 (D), AMPKα (E) and PPARα (F) in C, C+B, C+N, H, H+B, and H+N rats. Data expressed in arbitrary units normalized to β-Actin, shown as mean ± SEM. Means without a common letter in each data set significantly differ, P<0.05 and n=10/group. CTGF, connective tissue growth factor; MMP-2, matrix metalloproteinase-2; MCP-1, monocyte chemoattractant protein-1; TGF-β, transforming growth factor beta; AMPKα, adenosine monophosphate-activated protein kinase alpha; PPARα, peroxisome proliferator-activated receptor alpha.

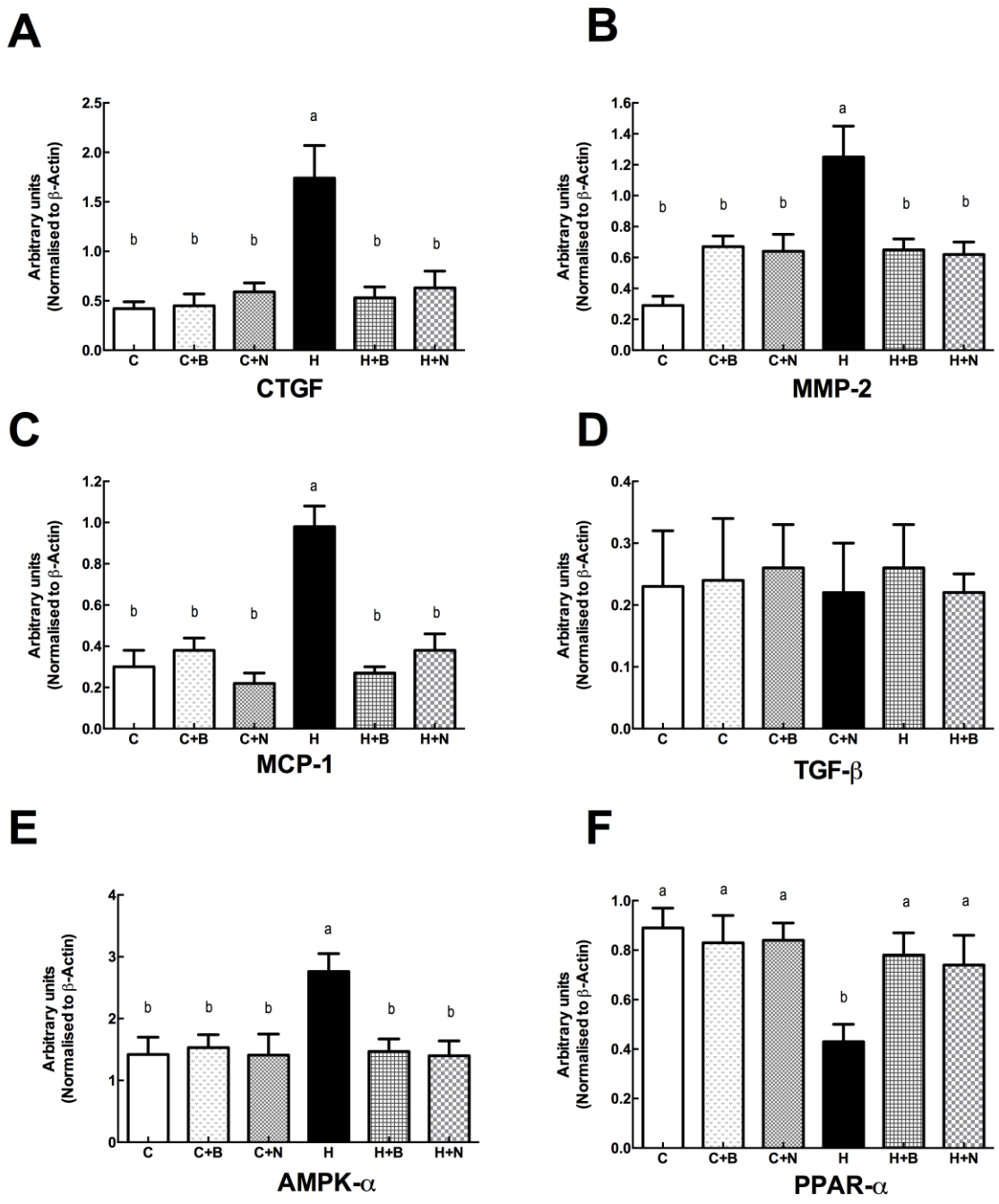


Figure 4. Hematoxylin and eosin staining of hepatocytes (original magnification ×20) showing inflammatory cells (marked as "in") and hepatocytes with fat vacuoles (marked as "fv") in C (A), C+B (B), C+N (C), H (D), H+B (E), and H+N (F) rats.

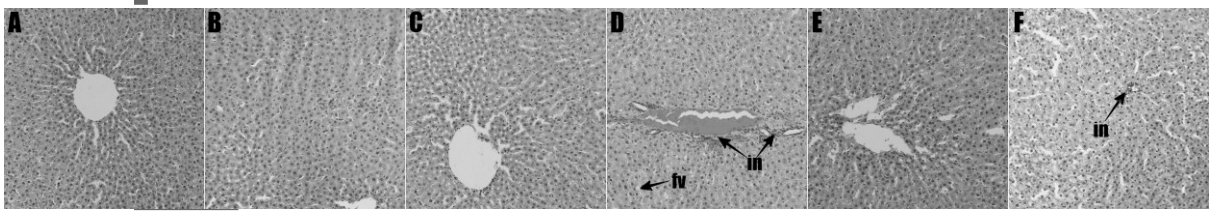
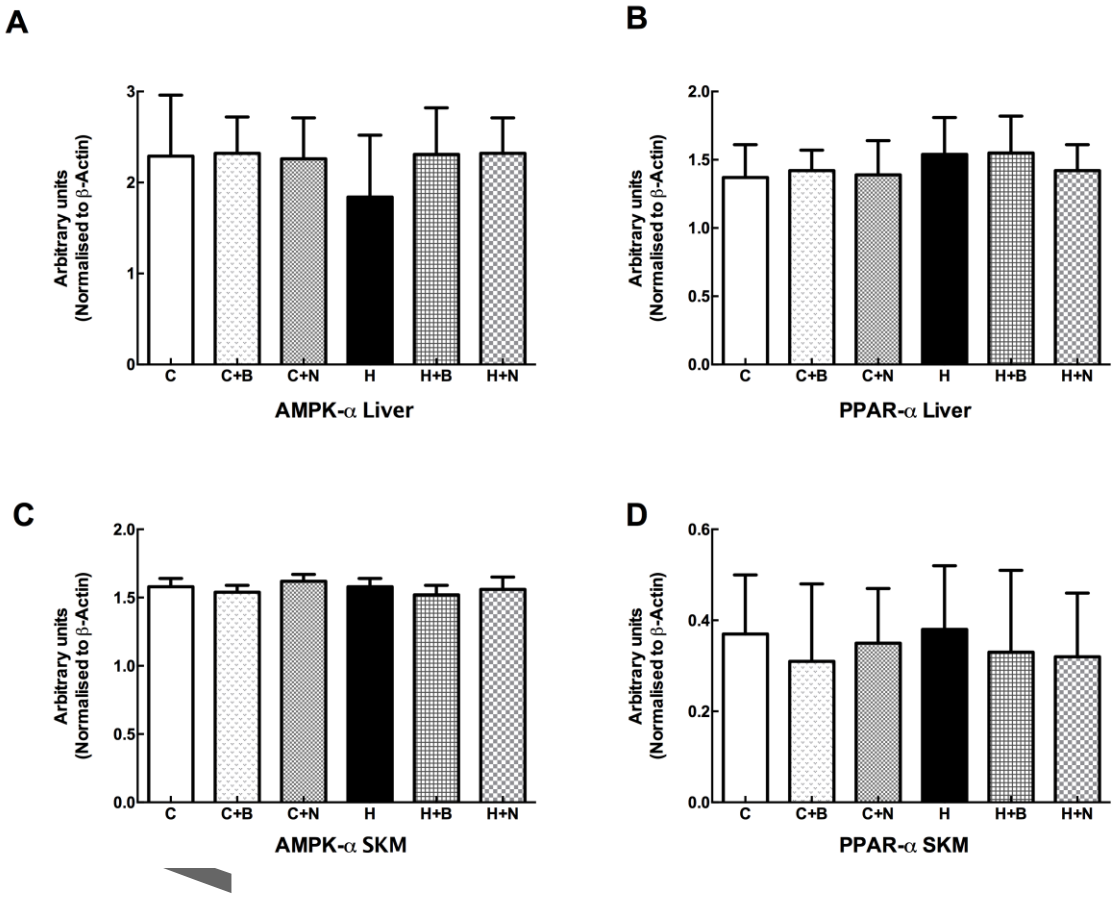


Figure 5. Hepatic and skeletal muscle mRNA expression of AMPKα and PPARα in C, C+B, C+N, H, H+B, and H+N rats. Data expressed in arbitrary units normalized to β-actin, shown as mean ± SEM. Means without a common letter in each data set significantly differ, P<0.05 and n=10/group. AMPKα, adenosine monophosphate-activated protein kinase alpha; PPARα, peroxisome proliferator-activated receptor alpha.



Graphical abstract-text

Rats fed a diet high in fat and carbohydrate developed cardiometabolic disorders. Beetroot juice,

which is a rich source of nitrate, reversed the diet-induced symptoms of metabolic syndrome in rats. Treatment of the rats with an equivalent dose of sodium nitrate to that contained in the juice replicated the beneficial effects of beetroot. The mechanisms underpinning the beneficial effects are likely to include prevention of infiltration of inflammatory cells to metabolic tissues including the heart and liver, as well as activation.

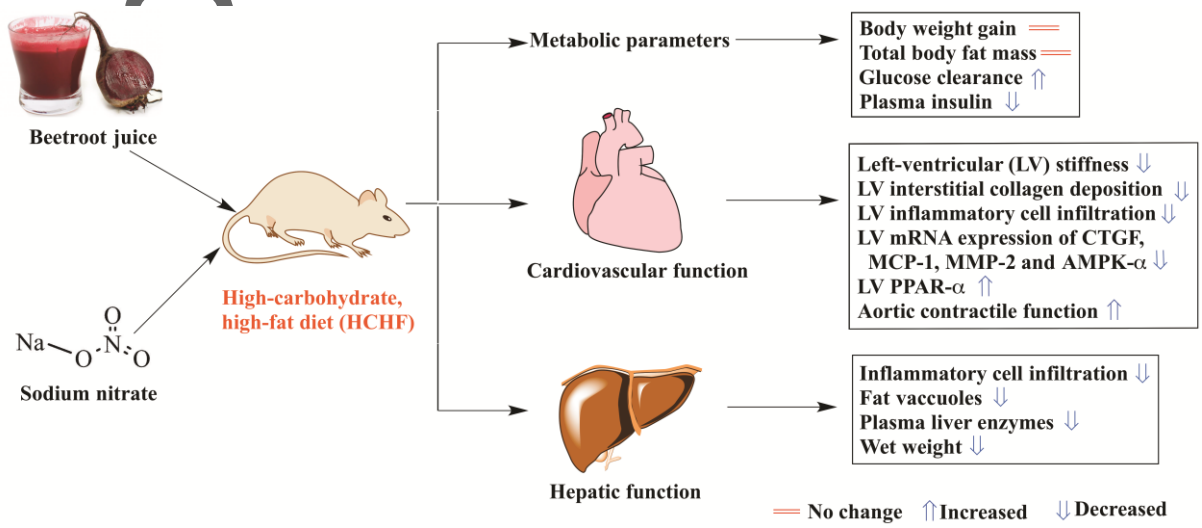


Table 1. Body composition, energy intake, oral glucose tolerance test, plasma insulin, and plasma biochemistry in C, C+B, C+N, H, H+B, and H+N diet-fed rats (n=10 rats/group).

Variable	C	C+B	C+N	H	H+B	H+N	P values		
							Diet	Treatment	Interaction
Body composition and energy intake									
Total body lean mass (g)	298.6 ±7.1[a]	271.8 ±6.9[b]	269.9 ±4.5[b]	302.1 ±8.9[a]	282.5 ±7.2[a]	285.1 ±8.4[a]	0.11	0.0027	0.72
Total body fat mass (g)	101.3 ±8.1[b]	104.3 ±8.6[b]	96.0 ±4.7[b]	186.6 ±20.2[a]	180.9 ±12.9[a]	189.4 ±11.3[a]	<0.0001	0.99	0.78
Energy intake (kJ/d)	393.5 ±15.1[b]	394.9 ±5.8[b]	399.4 ±8.6[b]	565.3 ±14.4[a]	553.9 ±7.5[a]	584.8 ±12.9[a]	<0.0001	0.28	0.51
Body weight gained (8-16 weeks) (%)	7.4 ±1.9[b]	6.8 ±0.8[b]	5.6 ±1.4[b]	15.1 ±0.9[a]	12.0 ±0.9[a]	11.2 ±1.5[a]	<0.0001	0.13	0.4

Glucose metabolism and plasma biochemistry									
OGTT-AUC (mmol/L min)	751.2 ±11.7[c]	670.1 ±7.5[d]	639.3 ±6.2[e]	827.4 ±12.9[a]	797.1 ±18.5[b]	695.3 ±12.2[d]	<0.0001	<0.0001	0.0154
Plasma insulin (µmol/L)	1.9 ±0.4[b]	2.0 ±0.5[b]	1.7 ±0.3[b]	4.1 ±0.5[a]	2.3 ±0.4[b]	2.1 ±0.4[b]	0.0416	0.0091	0.0242
ALP (U/L)	115.3 ±8.3[c]	136.9 ±6.6[c]	135.8 ±12.3[c]	325.8 ±27.6[a]	235.8 ±18.8[b]	228.3 ±17.1[b]	<0.0001	0.0488	0.001
ALT (U/L)	26.3 ±1.5[b]	26.4 ±1.1[b]	29.3 ±1.1[b]	34.3 ±1.6[a]	29.3 ±1.2[b]	30.1 ±1.4[b]	0.0007	0.17	0.0269
AST (U/L)	67.3 ±6.1[b]	60.4 ±1.9[b]	60.8 ±1.3[b]	87.5 ±3.0[a]	61.3 ±2.9[b]	63.9 ±3.6[b]	0.0064	<0.0001	0.0144
Total cholesterol (mmol/L)	1.9 ±0.1[b]	1.3 ±0.1[c]	1.5 ±0.1[c]	2.6 ±0.2[a]	1.4 ±0.1[c]	1.6 ±0.1[c]	0.0013	<0.0001	0.009
Triglycerides (mmol/L)	0.5 ±0.1[b]	0.4 ±0.1[b]	0.5 ±0.1[b]	1.8 ±0.3[a]	0.9 ±0.2[b]	0.8 ±0.1[b]	<0.0001	0.0036	0.0083
NEFA (mmol/L)	1.3 ±0.2[b]	1.3 ±0.1[b]	1.4 ±0.1[b]	5.4 ±0.6[a]	2.4 ±0.3[b]	2.5 ±0.3[b]	<0.0001	<0.0001	<0.0001

Each value is mean ± SEM.

[a,b,c,d,e]Means within a row with superscripts a, b, c, d and e differ significantly ($P < 0.05$).

*In all groups body-weight gained calculated as percentage of body weight increase from 8 weeks to 16 weeks. OGTT-AUC, oral glucose tolerance test-area under the curve; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; NEFA, non-esterified fatty acids.

Table 2. Cardiovascular structure and function in C, C+B, C+N, H, H+B, and H+N diet-fed rats (n=10 rats/group).

Variable	C	C+B	C+N	H	H+B	H+N	P values		
							Diet	Treatme	Interacti

								nt	on
Heart rate (bpm)	272 ±23[b]	271 ±9[b]	245 ±14[b]	365 ±24[a]	298 ±5[b]	241 ±35[b]	0.0308	0.0036	0.07
IVSd (mm)	1.9 ±0.1	1.8 ±0.0	1.9 ±0.0	2.0 ±0.1	1.8 ±0.0	2.0 ±0.0	0.16	0.017	0.61
LVIDd (mm)	6.9 ±0.2[b]	6.8 ±0.1[b]	6.4 ±0.2[b]	8.7 ±0.4[a]	6.4 ±0.2[b]	6.5 ±0.2[b]	0.001	<0.0001	<0.0001
LVPWd (mm)	1.7 ±0.1	1.8 ±0.0	1.8 ±0.0	1.8 ±0.0	1.8 ±0.1	1.8 ±0.0	0.57	0.72	0.72
IVSs (mm)	3.2 ±0.1	3.1 ±0.1	3.3 ±0.1	2.9±0.1	3.1 ±0.1	3.3 ±0.1	0.23	0.0393	0.23
LVIDs (mm)	3.8 ±0.2[b]	3.7 ±0.2[b]	3.8 ±0.2[b]	4.8 ±0.4[a]	3.8 ±0.2[b]	3.4 ±0.3[b]	0.24	0.0133	0.0178
LVPWs (mm)	2.9 ±0.1	2.8 ±0.1	2.8 ±0.1	2.9 ±0.1	3.0 ±0.1	3.1 ±0.1	0.0475	0.85	0.32
Fractional shortening (%)	48.5 ±2.1[b]	58.8 ±0.6[a]	54.3 ±1.2[a]	42.3 ±2.7[b]	55.5 ±1.0[a]	58.5 ±0.8[a]	0.14	<0.0001	0.0027
Ejection time (ms)	80.1 ±2.5[b]	93.2 ±2.1[a]	95.6 ±2.4[a]	72.9 ±3.9[b]	85.9 ±2.1[a]	88.4 ±2.0[a]	0.0014	<0.0001	1
Ejection fraction (%)	85.8 ±2.0	81.1 ±1.7	77.6 ±1.4	82.5 ±2.4	81.3 ±1.9	81.7 ±2.8	0.85	0.1	0.22
Diastolic volume (µL)	359 ±37[b]	340 ±24[b]	275 ±32[b]	688 ±66[a]	281 ±36[a]	292 ±33[a]	0.0061	<0.0001	<0.0001
Systolic volume (µL)	60 ±9[b]	58 ±9[b]	60 ±8[b]	121 ±22[a]	61±11[b]	51 ±16[b]	0.12	0.0282	0.0321
Stroke volume (µL)	309 ±33[b]	281 ±20[b]	215 ±27[b]	567 ±38[a]	219 ±31[b]	242 ±22[b]	0.0037	<0.0001	<0.0001

Cardiac output (mL/min)	88.7 ±13.6[b]	77.1 ±6.4[b]	62.8 ±9.6[b]	184.4 ±19.0[a]	65.5 ±10.0[b]	62.4 ±12.1[b]	0.0087	<0.0001	0.0001
Diastolic stiffness (k)	22.9±0.6[b]	23.1±0.4[b]	22.6±0.7[b]	28.6±0.7[a]	24.3±0.6[b]	23.8±0.9[b]	<0.0001	0.0008	0.0013
Estimated LV mass, Litwin (g)	0.8 ±0.1[b]	0.7 ±0.0[b]	0.8 ±0.0[b]	1.1 ±0.1[a]	0.8 ±0.0[b]	0.8 ±0.0[b]	0.0071	0.0035	0.0393
LV+septum wet weight (mg/mm tibial length)	17.8 ±0.5[b]	17.1 ±0.4[b]	15.3 ±0.5[c]	20.1 ±0.6[a]	17.6 ±0.4[b]	16.2 ±0.4[bc]	0.0023	<0.0001	0.15
Right ventricle wet weight (mg/mm tibial length)	4.6 ±0.5[ab]	4.1 ±0.1[b]	3.7 ±0.2[c]	5.6 ±0.4[a]	4.9 ±0.3[ab]	4.1 ±0.2[b]	0.0059	0.0015	0.62
Relative wall thickness	0.5±0.03[b]	0.5±0.02[b]	0.5±0.02[b]	0.6±0.03[a]	0.5±0.02[b]	0.5 ±0.01[b]	0.08	0.0497	0.0497
Systolic wall stress (mmHg)	87.7 ±7.0[b]	85.8 ±6.6[b]	83.4 ±6.5[b]	111.2 ±7.4[a]	82.4 ±7.3[b]	74.9 ±5.8[b]	0.49	0.0114	0.0483
LV developed pressure (mmHg)	54.7±3.4[ab]	62.4±5.1[a]	68.2±4.7[a]	44.7±3.6[b]	64.2±4.2[a]	66.8±3.8[a]	0.37	0.0004	0.38

(+)dP/dt (mmHg/S)	1147±75[a]	1229±67[a]	1197±66[a]	825±84[b]	1210±64[a]	1242±78[a]	0.1	0.0022	0.0334
(-)dP/dt (mmHg/S)	-705±52[a]	-785±56[a]	-758±47[a]	-473±47[b]	-767±50[a]	-783±55[a]	0.08	0.0006	0.034
Systolic blood pressure (mmHg)	129.4 ±2.5[b]	125.7 ±1.2[b]	122.6 ±1.7[b]	151.0 ±2.4[a]	133.5 ±2.2[b]	132.0 ±2.4[b]	<0.0001	<0.0001	0.0034

Each value is mean ± SEM.

[a,b,c]Means within a row with superscripts a, b and c differ significantly ($P < 0.05$).

C, corn-starch rich diet; H, high-carbohydrate, high-fat diet; B, beetroot; N, sodium nitrate; IVSd, interventricular septal end diastole; LVIDd, left ventricular internal diameter end diastole; LVPWd, left ventricular posterior wall end diastole; IVSs, interventricular septal end systole; LVIDs, left ventricular internal diameter end systole; LVPWd, left ventricular posterior wall end systole; dP/dt, left ventricular contractility.

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