

High sucrose/fat diet and isosorbide mononitrate increase insulin resistance, nitric oxide production and myocardial apoptosis in a hypertensive rat model

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Abstract. The present study aimed to investigate the association between insulin resistance (IR), nitric oxide (NO) production and myocardial apoptosis in a background of coexisting hypertension in a rodent animal model. A hypertensive rat model was established by feeding Wistar and spontaneously hypertensive rats (SHR) with a high sucrose/fat (HSF) diet for 12 weeks, in conjunction with isosorbide mononitrate (ISMN). Increased IR, NO content, apoptotic gene and protein expression, and morphological alterations within rat myocardium were evaluated. Following a total of 12 weeks of feeding with HSF and ISMN resulted in increased IR and NO content within the myocardial tissue of Wistar and SHR rats. HSF and ISMN activated myocardial apoptosis by downregulating the gene transcription and protein expression levels of the anti-apoptotic B-cell lymphoma 2 (Bcl-2), and increasing the pro-apoptotic Bcl-2 associated X protein. Apoptosis was demonstrated by DNA fragmentation in terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling assay. In all experiments, the combination of HSF and ISMN

was associated with more pronounced effects, indicating the possible synergistic effects. In addition, the correlation analysis in the Wistar rats fed with HSF only, revealed a positive association between NO production and IR. The results of the present study indicated that HSF and ISMN simultaneously increased IR, NO production and myocardial apoptosis in the hypertensive rat model, and may therefore contribute to investigations into the long-term clinical use of ISMN in hypertensive patients.

Introduction

Metabolic syndrome (MS) is a constellation of pathologic conditions including hypertension, hyperglycemia, dyslipidemia and abdominal obesity (1). As one of the core components of MS, hypertension further leads to the development of atherosclerotic cardiovascular disease (ASCVD) by inducing myocardial ischemia and apoptosis. In fact, according to the 1992 Framingham Study, hypertension accounted for ~25% of cardiac failures (2), with a morbidity rate of ≥50% in elderly patients (3).

In patients with hyperglycemia, particularly with type II diabetes mellitus, serum insulin levels may be normal or abnormally increased compared with the reference range. This is known as insulin resistance (IR) (4). Hyperglycemia and IR are also considered core components of MS, serving a central role in the progression of ASCVD (5).

Isosorbide mononitrate (ISMN) is an organic nitrate used for the prevention and treatment of ASCVD. ISMN generates exogenous nitric oxide (NO) to expand coronary arteries and improve the functions of endothelial cells. The resulting dilation of coronary vessels improves oxygen supply to the myocardium (6). However, studies have also demonstrated that NO may induce myocardial apoptosis (7). In one study, Wang *et al* (8) reported that ISMN synergized with aspirin in activating the NO signaling and activated apoptosis in human colon cancer cells.

To the best of the author's knowledge, a direct investigation into the association between IR, NO content and myocardial apoptosis in a background of coexisting hypertension in

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a rodent animal model has not yet been conducted. In the present study, a hypertensive model was established by feeding Wistar and spontaneously hypertensive rats (SHR) with a high sucrose/fat (HSF) diet, in conjunction with ISMN. ISMN is extensively prescribed for patients with ASCVD. The present study also aimed to address the pathophysiological effects from long-term use of ISMN in hypertensive patients.

Materials and methods

Hypertensive animal model. The hypertensive animal model was established with 14-week-old male SHR (Beijing Wei Tong Li Hua Experimental Animal Technology Co., Ltd, China) and 14-week-old male Wistar rats (Experimental Animal Center of Henan, Henan, China). Rats were housed in conventional cages (5 rats/cage) with free access to food and water at a controlled temperature ($23\pm 2^{\circ}\text{C}$) and humidity ($55\pm 5\%$) under a 12 h light/dark cycle starting at 6:00 a.m. Body weight and caloric intake were recorded weekly.

A total of 40 male Wistar rats (body weight, 394.45 ± 10.19 g) were randomly divided into 4 groups (1-4); 40 SHR (body weight, 393.14 ± 11.12 g) were also randomly divided into four groups (5-8). Group 1 and 5 rats were fed a normal diet. Group 2 and 6 rats were fed a HSF diet. Group 3 and 7 rats were fed a normal diet supplemented with ISMN. Group 4 and 8 rats were fed with the HSF and ISMN. The HSF diet was composed of 79% normal diet, 10% sucrose, 5% lard, 5% cholesterol, and 1% lithocholic acid. The experimental protocol followed the guidance for the Care and Use of Laboratory Animals (US National Institutes of Health, no. 85-23) (9) and the guidelines of the Animal Care and Use Committee of Zhengzhou University. The present study was approved by the Ethics Review Committee of Second Affiliated Hospital of Zhengzhou University.

Sampling of arterial blood and myocardium. A total of 12 weeks post-feeding, the rats were anesthetized by intraperitoneal injection of chloral hydrate (300 mg/kg, C8383; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Arterial blood (1 ml) was drawn by carotid artery intubation; arterial blood, instead of peripheral (venous) blood is technically more convenient and allows a larger blood volume for subsequent experiments. The blood was centrifuged at $500 \times g$ at room temperature for 5 min and the plasma was snap frozen in liquid nitrogen and stored at -20°C until use. Subsequently, the rats were euthanized by supplementary intraperitoneal injection of sodium pentobarbital (70 mg/kg, 1507002; Sigma-Aldrich; Merck KGaA) approved by the guidelines of the Animal Care and Use Committee of Zhengzhou University. The hearts were surgically dissected and immersed in ice-cold saline to remove blood. A total of 4 sections of full-thickness myocardium were taken from the left ventricle. Of these, one section was used immediately for the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. The remaining sections were snap frozen in liquid nitrogen and preserved at -80°C for the NO assay and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Snap freezing is the technique of rapid sample freezing with liquid nitrogen and maintains tissue sample integrity and delays the actions of proteases and nucleases that inhibit degradation of RNA or proteins used in molecular assays.

TUNEL assay. The TUNEL assay was performed on cardiomyocytes seeded on chamber slides as previously described (10,11). In brief, 2 days following isolation, the primary rat cardiomyocytes were incubated with 1% pericardial fluid for 48 h at 37°C . The cells were fixed in 10% neutral buffered formalin for 10 min at room temperature. The TUNEL assay was performed on fixed cardiomyocytes with an *in-situ* Apoptosis Detection kit, according to the manufacturer's protocols (MK500; Takara Bio Inc., Otsu, Japan). Individual nuclei were observed and images were captured at $\times 400$ with a standard Olympus bright field microscopy (Olympus Corporation, Tokyo, Japan) for quantitative analysis.

NO assay. NO contents in rat myocardium were determined using the nitric acid deoxidize enzyme method with a commercial assay kit according to the manufacturer's protocol (A012; Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

RT-PCR. A total of 100 mg frozen rat myocardium was homogenized in 1 ml TRIzol® (15596018; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total RNA was extracted with chloroform, precipitated with isopropyl alcohol and ethanol wash, according to the manufacturer's protocol. Total RNA was dissolved in nuclease-free water with the concentration determined by UV spectroscopy at a wavelength of 260 nm. RT was conducted according to the manufacturer's protocols using a high capacity cDNA reverse transcription kit (4368814; Thermo Fisher Scientific, Inc.). The semi-quantitative PCR reactions were carried out on an Eppendorf thermal cycler with a Taq PCR kit (New England BioLabs, Inc., Ipswich, MA, USA) and PCR primers listed as below: B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) forward, 5'GGGTGGTTGCCCTTTTCTAC3' and reverse, 5'GGTGAGTGAGGCAGTGAGGA3'; BCL-2 forward, 5'CTGTGGTCCACCTGACCCTC3' and reverse, 5'GGCATCCCAGCCTCCGTTAT3'; GAPDH forward, 5'TCAACGGCACAGTCAAGG3' and reverse, 5'GGGTAGGAACACGGAAGG3'. The PCR reaction included the following thermocycling conditions: Initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at an oligo-specific temperature (Bax, 52°C ; BCL-2, 58°C ; GAPDH, 55°C) for 30 sec, and extension at 72°C for 30 sec. The PCR products were analysed by 1% agarose gel electrophoresis, visualized using ethidium bromide and quantified using densitometry with ImageJ software bundled with 64-bit Java (1.6.0_24; National Institutes of Health, Bethesda, MD, USA). GAPDH was used as the internal control.

Western blotting. Total proteins from rat myocardial tissue were extracted with radioimmunoprecipitation assay lysis buffer supplemented with protease and phosphatase inhibitors (MSSAFE-5VL; Sigma-Aldrich, Merck KGaA). Protein concentration was determined using the standard Bicinchoninic Acid protein assay following the supplier's protocol (Thermo Fisher Scientific, Inc.). Equal amounts (typically 30 μg /lane) of proteins were resolved by 4-12% SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in Tris buffer saline containing 0.2% Tween-20 (TBST) at room temperature for 1 h and incubated with primary antibodies against Bcl-2 (1:1,000), Bax (1:1,000), GAPDH (1:2,500) at 4°C overnight. The membranes

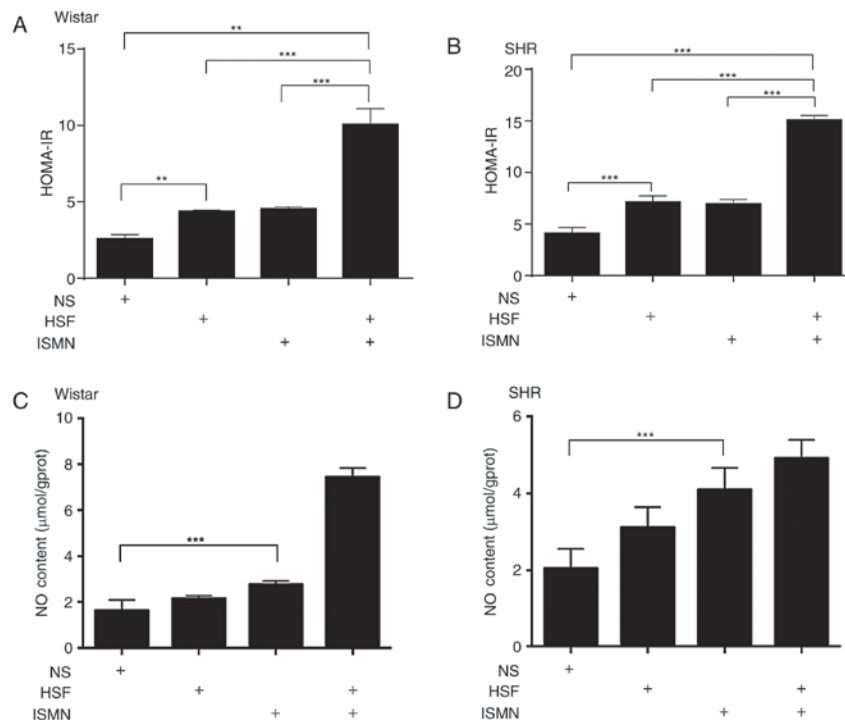


Figure 1. HSF and ISMN result in HOMA-IR and NO content increase in the rat myocardial tissue. Following 12 weeks of feeding with HSF, ISMN or a combination, resulted in increased HOMA-IR in the arterial blood of (A) Wistar rats and (B) SHR, with possible synergistic effects between HSF and ISMN. HSF alone exhibited no notable impact on NO production in (C) Wistar rats or (D) SHR; however, HSF increased the myocardial NO production in the two strains of rats. ISMN alone, or in combination with HSF resulted in pronounced increases in NO content in Wistar and SHR rats compared with the NS. Data were expressed as mean \pm standard deviation (n=10). **P<0.01, ***P<0.001. HOMA-IR, higher insulin resistance; HSF, high sucrose/fat diet; ISMN, isosorbide mononitrate; NO, nitric oxide; NS, normal diet; SHR, spontaneously hypertensive rats.

were washed with TBST 3 times then incubated with horse-radish peroxidase-conjugated secondary antibodies (1:2,500) at room temperature for 2 h. All primary antibodies (sc-20067, sc-56015, sc-516142) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Secondary antibodies were acquired from Zymed Laboratories (31460; Thermo Fisher Scientific, Inc.). The western blot bands were visualized using enhanced chemiluminescence reagent (cat. no. RPN2232; Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) and quantified using densitometric analysis with ImageJ software bundled with 64-bit Java (1.6.0_24; National Institutes of Health).

IR index. The homeostatic model assessment of higher insulin resistance (HOMA-IR) was used to quantify the IR as follows: Fasting blood glucose (mmol/l) \times fasting insulin (mIU/l)/22.5. The fasting glucose was determined by our hospital chemistry laboratory using the oxidase test. The insulin was similarly determined using the 2-site electrochemiluminescent insulin immunoassay.

Statistical analysis. Data were expressed as the mean \pm standard deviation. Comparison between two groups was analyzed with two-sample t-test. Comparison of data in more than two groups was performed with one-way analysis of variance, followed by Fisher's least significant difference comparison-t-test. Comparison and Pearson's correlation analysis were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

HSF and ISMN result in increased HOMA-IR and NO content in myocardial tissue. In Wistar and SHR rats, 12 weeks of HSF feeding resulted in significantly increased HOMA-IR compared with the normal diet (Fig. 1A and B). Alterations largely consistent with the elevated IR in the two rat strains were also observed for the blood glucose (data not shown). HSF feeding had no notable impact on the NO production in myocardial tissue (Fig. 1C and D). Supplementing ISMN in the two rat species fed with HSF induced an increase in HOMA-IR levels. In addition, ISMN significantly increased the NO content in myocardial tissue (Fig. 1C and D). A combination of HSF and ISMN was associated with more pronounced increases in the HOMA-IR in Wistar and SHR rats, indicating the possible synergistic effects of HSF and ISMN on HOMA-IR. Although not significant by statistical analysis, HSF also appeared to visually augment the activity of ISMN in producing NO in myocardial tissue.

HSF and ISMN activate myocardial apoptosis. Subsequently, myocardial apoptosis was analyzed by quantifying the mRNA transcription and protein expression of key apoptotic components. RT-qPCR with total RNA extracted from two rat strains revealed that HSF and ISMN feeding resulted in reduced transcription of the anti-apoptotic gene Bcl-2 (Fig. 2A and B), and increased the transcription of the pro-apoptotic gene Bax (Fig. 2C and D). The combination of HSF and ISMN appeared to have synergistic effects. At the protein level, similar alterations were observed. HSF and

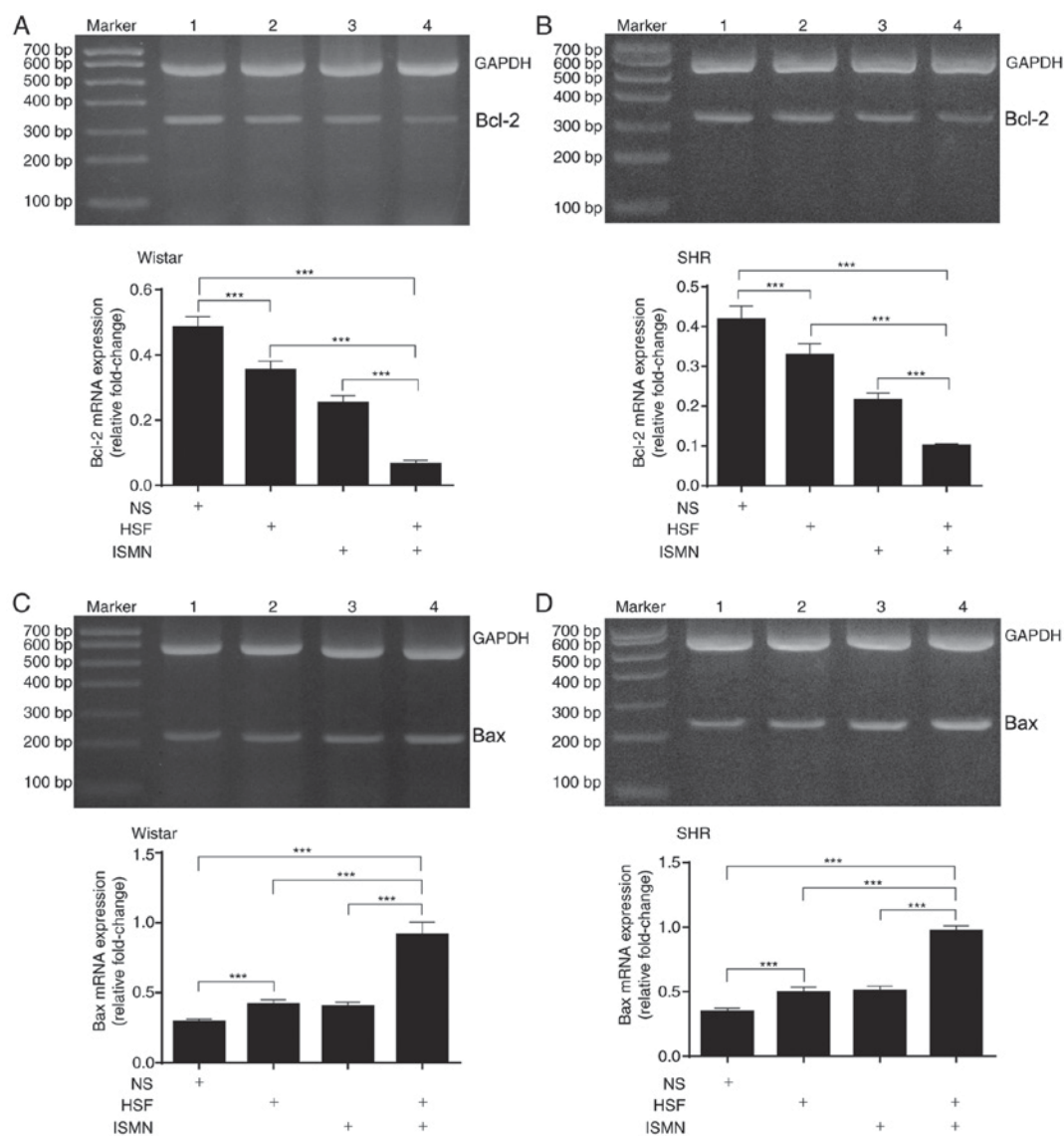


Figure 2. HSF and ISMN activate the mitochondrial apoptotic pathway in rat myocardium by affecting the transcription of Bcl-2 and Bax. Following 12 weeks of feeding with HSF and ISMN, transcription levels of the anti-apoptotic gene BCL-2 reduced within (A) Wistar rats and (B) SHR. Representative images of agarose gel electrophoresis of the quantitative polymerase chain reaction products are also presented. Transcription of the pro-apoptotic gene Bax increased in (C) Wistar rats and (D) SHR. The combination of HSF and ISMN appeared to have synergistic effects. GAPDH served as the internal control. Lane 1, normal diet; lane 2, HSF; lane 3, ISMN; lane 4, combination. Bar graph data were expressed as mean \pm standard deviation ($n=10$). *** $P<0.001$. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; HSF, high sucrose/fat diet; ISMN, isosorbide mononitrate; NS, normal diet; SHR, spontaneously hypertensive rats.

ISMN feeding suppressed the protein expression of Bcl-2 and activated Bax, with a notable synergism between HSF and ISMN (Fig. 3).

Finally, myocardial apoptosis was analyzed via a TUNEL assay, which detected DNA fragmentation, a characteristic hallmark of apoptosis. The immunohistochemical staining of the myocardium revealed enhanced apoptosis within rats fed with the HSF or ISMN diet (Fig. 4). The Wistar and SHR rats fed with the combinational diet of HSF and ISMN demonstrated more intense staining in the TUNEL assay.

NO production in rat myocardium is positively associated with HOMA-IR. To understand the association between NO content and HOMA-IR, Pearson's correlation test was performed. In group 2 Wistar hypertensive rats fed with HSF, a positive association between the NO content and HOMA-IR was observed (Fig. 5).

Discussion

NO serves important roles in diverse physiological and pathological processes, including vasodilatation, oxidative stress and inflammation (6). NO produced predominantly by the vascular endothelium, leads to direct relaxation of the vascular smooth muscles (12). The dilation of veins promotes peripheral pooling of blood and reduces venous return to the right-side of the heart, thereby lessening preload (12). Arteriolar relaxation, however, reduces systemic vascular resistance and systolic arterial pressure (12). NO also directly dilates the coronary arteries thereby increasing the blood flow to myometrium. ISMN, an exogenous NO inducer, is widely prescribed for the prophylactic treatment of angina pectoria and early management of myocardial infarction (13).

In the present study, SHR and Wistar rats served as the experimental model. SHR rats are widely used as an animal

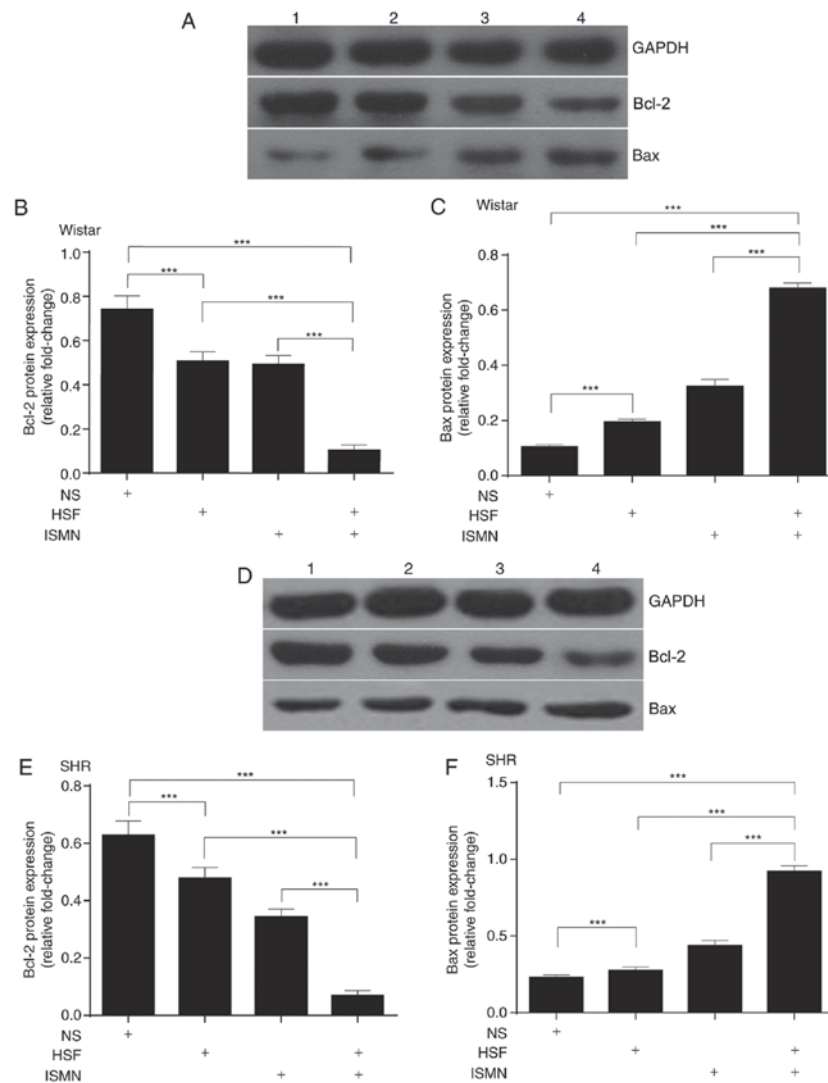


Figure 3. HSF and ISMN induce the mitochondrial apoptotic pathway by altering the protein expression levels of Bcl-2 and Bax. Following 12 weeks of feeding with HSF and ISMN, Bcl-2 expression was suppressed and the expression of Bax was activated. (A) Representative image and quantitative analysis of (B) Bcl-2 and (C) Bax protein expression levels as revealed by western blotting in Wistar rats. (D) Representative image and quantitative analysis of (E) Bcl-2 and (F) Bax protein expression levels as revealed by western blotting in SHR. There was a notable synergism between HSF and ISMN. Representative western blotting data were presented in (A and D). GAPDH served as the loading control. Lane 1, normal diet; lane 2, HSF; lane 3, ISMN; lane 4, combination. Bar graph data were expressed as mean \pm standard deviation (n=10). ***P<0.001. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; HSF, high sucrose/fat diet; ISMN, isosorbide mononitrate; NS, normal diet; SHR, spontaneously hypertensive rats.

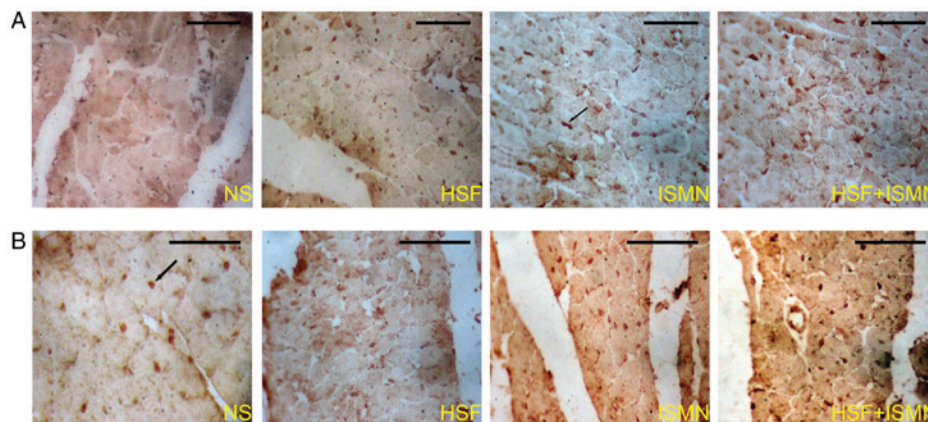


Figure 4. HSF and ISMN activate apoptosis in the hypertensive rat myocardium. Representative light-microscopic photographs (magnification, x400) of the TUNEL assay demonstrated that HSF and ISMN activated apoptosis in the hypertensive rat myocardium. Apoptotic cell nuclei were stained brown (indicated by arrows). The (A) Wistar and (B) SHR rats fed with the combination diet revealed more intense staining in the TUNEL assay. Scale bars represent 50 μ m. HSF, high sucrose/fat diet; ISMN, isosorbide mononitrate; NS, normal diet; SHR, spontaneously hypertensive rats; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling.

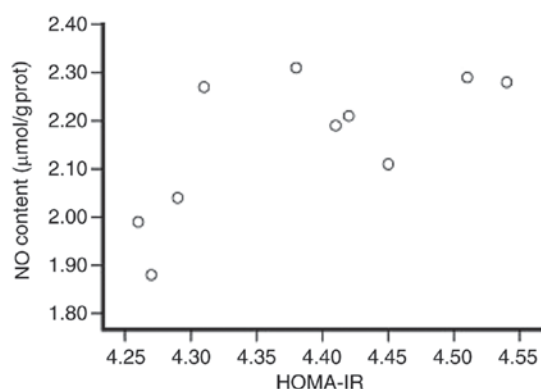


Figure 5. NO production in the rat myocardium is positively correlated with HOMA-IR. In group 2 Wistar hypertensive rats fed with HSF, there was a positive correlation between the NO content and HOMA-IR. $n=10$, $r=0.684$, $P=0.029$. HOMA-IR, higher insulin resistance; NO; nitric oxide.

model of essential hypertension and cardiovascular disease. They are derived from the parental strain Wistar rats. Generally, rats are favored over mice for a more assimilated physiology to humans, making them better suited for the study of pathological conditions, including cardiovascular disease. Rats are also technically more advantageous, with their larger size allowing advanced surgical procedures and larger volume of body fluids and tissue for adequate experimental readouts. The hypertensive rat model of the present study revealed that the basal myocardial NO content in SHR rats was increased compared with in the Wistar rats. The elevated NO level in SHR rats may be attributed to the increased protein-bound dinitrosyl nonheme iron complexes, which release NO to the peripheral circulation to combat the hypertensive state (14). This protective mechanism may be compensatory to maintaining the systemic blood pressure at low levels (12). In addition, increased activity of NO synthase (NOS) III and augmented expression of NOS II have been reported in the cardiac and aortic endothelia (15). These two enzymes may regulate the vasoreactivity in the SHR rats.

The data of the present study demonstrated that feeding with HSF or ISMN increased the proapoptotic protein Bax and suppressed the anti-apoptotic Bcl-2. This was accompanied by the induction of myocardial apoptosis, as demonstrated by the TUNEL assay. Excessive production of NO by HSF or ISMN feeding has been reported to mediate the apoptotic cell death of myocardium via the cyclic guanosine monophosphate pathway, and generate peroxynitrite to damage myocardium by reacting with the superoxide anion (16,17).

In eukaryotes, there are two primary apoptotic pathways: The extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The Bcl-2 family of proteins regulate apoptosis by controlling the mitochondrial permeability. The anti-apoptotic proteins, Bcl-2 and Bcl-xL, reside in the outer mitochondrial wall and inhibit cytochrome *c* release. Conversely, the cytoplasmic pro-apoptotic protein Bax translocates to mitochondria following apoptotic signaling, promoting the release of cytochrome *c*. Upon release, cytochrome *c* associates with apoptosis protease activating factor 1 to form the apoptosome, that recruits and activates procaspase-9. This is followed by activation of executioner caspases including caspase-3 or -7 and terminal events proteolysis and

DNA fragmentation. It has been reported that NO promotes apoptosis by activating the mitochondria-dependent apoptotic cell death, which suggests the involvement of tumor suppressor p53 as a target during cell death execution (18).

It remains uncertain whether IR, as a core component of the metabolic syndrome, directly leads to myocardial apoptosis. The present study reported that myocardial apoptosis was significantly augmented in the hypertensive rats exposed to HSF or ISMN. This was accompanied by a greater degree of IR; thus, it may be assumed that there was a direct association between IR and myocardial apoptosis. The underlying mechanism may be too complex for the scope of the present study; the causal association was not examined. However, a number of earlier studies have suggested that certain pathological processes, including oxidative stress, inflammation, endothelial dysfunction and metabolic imbalance resulting from IR may directly damage myocardial cells, resulting in myocardial apoptosis and infarction (19-22). In particular, IR may induce systemic inflammatory factors, including C-reactive protein, tumor necrosis factor- α and interleukin-6, which initiate and aggravate atherosclerosis, leading to myocardial ischemia and apoptosis. Furthermore, IR has been reported to directly contribute to the pathogenesis of hypertension and the subsequent myocardial apoptosis. A possible theory of this causal association is the secondary hyperinsulinemia of IR, which enhances the ability of kidneys to reabsorb sodium and water, resulting in hypertension. The correlation analysis of the present study demonstrated a positive correlation between NO content within the myocardium of rats fed with HSF and the degree of HOMA-IR. This may be explained by the secondary hyperinsulinemia in IR, which induces endogenous NO secretion. A few studies have also suggested that IR was associated with an elevation in skeletal muscle inducible NOS (iNOS) (23-25), which may produce large amounts of NO. NO may alter the S-nitrosation of proteins involved in insulin signal transduction. S-nitrosation of insulin receptor β -subunit and protein kinase B may impair kinase activities, whereas S-nitrosation of insulin receptor substrate 1 reduces the tissue expression.

The present study may be limited by the capability of a rat model to efficiently represent a human disease. Furthermore, dynamic alterations in blood pressure were not monitored due to technical challenges. The conventional tail-cuff method requires special technical expertise and is disregarded by certain experts in the cardiovascular field due to the artefactual results from the physical restraint of animals and human influence (26). The gold-standard blood pressure measurement; however, requires the use of implanted telemetry. This technology is expensive requiring an elaborate technical setup. Various clinical studies have suggested that with a long-term application of ISMN, endothelial functions of patients may be compromised (27). The short duration (12 weeks) of the present study may not recapitulate the long-term and chronic alterations within the myocardium.

In conclusion, the findings of the present study suggested that HSF- and ISMN-feeding in Wistar and SHR rats may simultaneously induce IR and increase NO content in the myocardium. This process was accompanied by the activation of the mitochondrial death cascade and apoptosis in the myocardium.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

TL, BB and HW designed the experiments. TL, DJ and FM carried out all the experiments. TL and MS analyzed the data and wrote the manuscript. CT provided advice and guidance regarding analysis of data.

Ethics approval and consent to participate

The experimental protocol followed the guidance for the Care and Use of Laboratory Animals (US National Institutes of Health, no. 85-23) and the guidelines of the Animal Care and Use Committee of Zhengzhou University. The present study was approved by the Ethics Review Committee of Second Affiliated Hospital of Zhengzhou University (Zhengzhou, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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