นิพนธ์ต้นฉบับ • Original Article

สารสกัดจากกระเจี๊ยบแดงบรรเทาการทำงานที่ผิดปกติของชั้นเอนโดทีเลียมของหลอดเลือด โดยเพิ่มชีวปริมาณออกฤทธิ์ของไนตริกออกไซด์ ในหนูแรทที่มีภาวะดื้ออินซูลินเนื่องจากได้รับอาหารที่มีน้ำตาลฟรุกโทสสูง

สราวุธ บรรบุผา[1], ปาริฉัตร ประจะเนย์[2,3], เทวฤทธิ์ เบิกบาน[2], ยุพา คู่คงวิริยพันธุ์[1,3], พวงรัตน์ ภักดีโชติ[1,3*]
[1]ภาควิชาสรีรวิทยา [2]ภาควิชากายวิภาคศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ขอนแก่น
[3]กลุ่มวิจัยหัวใจและหลอดเลือด มหาวิทยาลัยขอนแก่น ขอนแก่น

Hibiscus sabdariffa L. Extract Alleviates Vascular Endothelial Dysfunction by Enhancing Nitric Oxide Bioavailability in High-Fructose Diet Induced Insulin Resistance Rats

Sarawoot Bunbupha[1], Parichat Prachaney[2,3], Thewarid Berkban[2], Upa Kukongviriyapan[1,3], Poungrat Pakdeechote[1,3*]
[1]Department of Physiology, [2]Department of Anatomy, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[3]Cardiovascular Research Group, Khon Kaen University, Khon Kaen, Thailand

หลักการและวัตถุประสงค์: การศึกษานี้ทดสอบผลของสารสกัดจากกระเจี๊ยบแดงต่อการทำงานของชั้นเอนโดทีเลียมของหลอดเลือด และกลไกที่เกี่ยวข้องในหนูแรทที่มีภาวะดื้ออินซูลินเนื่องจากได้รับอาหารที่มีน้ำตาลฟรุกโทสสูง

วิธีการศึกษา: หนูแรทเพศผู้ได้รับอาหารที่มีน้ำตาลฟรุกโทสสูงเป็นเวลา 18 สัปดาห์ เพื่อเหนี่ยวนำให้เกิดภาวะดื้ออินซูลิน ทำการป้อนสารสกัดจากกระเจี๊ยบแดง (200 หรือ 500 มก./กก./วัน) ใน 4 สัปดาห์สุดท้ายของการทดลอง เมื่อสิ้นสุดการทดลองทำการประเมินการทำงานของหลอดเลือด และตัวบ่งชี้ถึงเปลี่ยนแปลงของเมแทบอลิก

ผลการศึกษา: สารสกัดจากกระเจี๊ยบแดง (200 และ 500 มก/กก.) บรรเทาความทนทานต่อน้ำตาลบกพร่อง ลดควา000มดันเลือด และฟื้นฟูการทำงานของชั้นเอนโดทีเลียมของหลอดเลือด ($p<0.05$) นอกจากนี้สารสกัดจากกระเจี๊ยบแดง (500 มก/กก.) สามารถเพิ่มชีวปริมาณออกฤทธิ์ของไนตริกออกไซด์ โดยเพิ่มระดับพลาสมา NOx เพิ่มการแสดงออกของโปรตีน eNOS รวมทั้งลดการสร้าง $O_2^{\bullet-}$ ในหนูทดลองที่มีภาวะดื้ออินซูลิน ($p<0.05$)

Background and Objectives: This study evaluated the effect of *Hibiscus sabdariffa* extract (HSE) on vascular endothelial function and mechanism involved in insulin resistance rats induced by high-fructose diet (HFD).

Methods: Male Sprague-Dawley rats were fed with HFD for 18 weeks to induce insulin resistance. Oral administration of HSE (200 or 500 mg/kg/day) was performed in the last four weeks. Vascular function and metabolic parameters were evaluated.

Results: HSE (200 and 500 mg/kg) improved glucose tolerance, reduced blood pressure, and restored vascular endothelial dysfunction in rats fed with HFD ($p < 0.05$). Moreover, HSE 500 mg/kg enhanced nitric oxide bioavailability by restoring plasma NOx level and eNOS expression, and reducing $O_2^{\bullet-}$ production in insulin resistance rats ($p < 0.05$).

Conclusion: Our results suggest that HSE alleviated vascular endothelial dysfunction in HFD fed rats that was associated with increasing in NO bioavailability.

Keywords: *Hibiscus sabdariffa* L., insulin resistance, NO bioavailability

**Corresponding Author: Poungrat Pakdeechote, Department of Physiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. E-mail: ppoung@kku.ac.th*

สรุป: ผลการศึกษาแสดงให้เห็นว่าสารสกัดจากกระเจี๊ยบแดงบรรเทาการทำงานที่ผิดปกติของชั้นเอนโดทีเลียมของหลอดเลือด ในหนูแรทที่ได้รับอาหารที่มีน้ำตาลฟรุกโทสสูง ผลดังกล่าวมีความสัมพันธ์กับการเพิ่มชีวปริมาณออกฤทธิ์ของไนตริกออกไซด์

คำสำคัญ: กระเจี๊ยบแดง, ภาวะดื้ออินซูลิน, ชีวปริมาณออกฤทธิ์ของไนตริกออกไซด์

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Introduction

High fructose diet-induced insulin resistance and metabolic syndrome has been widely accepted as an animal model for metabolic and cardiovascular abnormalities that closely mimics characteristics of metabolic syndrome in humans. Recent findings supported that rats fed with a high-fructose diet (HFD) induces a well characterized metabolic syndrome, generally resulting in insulin resistance, dyslipidemia and hypertension[1]. Endothelial dysfunction and nitric oxide (NO) deficiency caused by decreased eNOS are implicated in the pathogenesis of hypertension. Several lines of evidence showed that HFD-induced insulin resistance is associated with vascular endothelial dysfunction and resulting hypertension[2,3].

Hibiscus sabdariffa L. (Malvaceae, common name Roselle), a native tropical plant, is widely cultivated in Thailand and commonly used as soft drinks and medicinal herbs. Several previous studies have shown a variety of biological effects of *Hibiscus sabdariffa* L. extract (HSE) such as antioxidant, antihypertensive, antihyperlipidemia, and antihyperglycemia properties[4,5]. The several antioxidant compounds isolated from the flowers of *Hibiscus sabdariffa* L. include anthocyanins, gallic acid, hibiscus acid and protocatechuic acid[4-6]. Hirunpanich and coworkers found that HSE has antioxidant activity by reducing thiobarbituric acid reactive substances (TBARs) formation in hypercholesterolemic rats[7]. Additionally, previous study demonstrated that HSE reduce blood pressure and improve endothelium-dependent vasorelaxation in HFD-induced insulin resistance rats[8].

Although a wide range of potentially therapeutic effects of HSE have been reported, the underlying mechanism of HSE to restore vascular endothelial function in HFD-fed rats remain unknown. Therefore, the aim of this study was to investigate the effect of HSE on acetylcholine (ACh)-induced vasorelaxation and blood pressure in HFD-fed rats. To clarify the underlying mechanism, NO level, ROS production, and protein eNOS expression were measured.

Materials and Methods

Chemicals

Ethylenediaminetetraacetic acid (EDTA), N-(1-Napthyl) ethylenediamine dihydrochloride (NED), and sulfanilamide were obtained from Sigma-Aldrich Corp. (St Louis, MO, USA). Nitrate reductase, nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate disodium and glucose-6-phosphate dehydrogenase were obtained from Roche Applied Sciences (Mannheim, Germany). All chemicals used were of analytical grade quality.

Preparation of HSE

The HSE was supplied by Applied Thai Traditional Medicine Centre, Thammasart University, Prathumthani, Thailand. Fresh calyces of *Hibiscus sabdariffa* L. collected from Khoun Meed District, Songkhla Province, Thailand. The plant specimen (No. SKP 1090819) was deposited in the herbarium of Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The calyces were dried at 50°C and then extracted using water. In brief, the calyces of

Hibiscus sabdariffa L. (5 kg) were boiled in water (30 L) for 15 min then the water extract was filtered through nylon cloth and then dried using a spray dry machine. The yield (calculated on the dried powder extract) was 4.6% of the fresh calyces weight and was 37.4% of dried calyces weight. Dried HSE was then packed in tight containers and kept at 4-6°C until used.

Animals and experimental protocol

Male Sprague-Dawley rats (200–220 g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. They were housed at 25 ± 2°C with a 12-h dark–light cycle at Northeast Laboratory Animal Center, Khon Kaen University, Khon Kaen, Thailand. All procedures were complied with the standards for the care and use of experimental animals and approved by the Animal Ethics Committee of Khon Kaen University (AEKKU 41/2551). The animals were randomly assigned to 5 groups of 7 rats each: Group I. Control + vehicle (distill water, 0.15 mL/100 g BW), Group II. Control + HSE (500 mg/kg/day), Group III. HFD + vehicle (distill water, 0.15 mL/100 g BW), Group IV. HFD + HSE (200 mg/kg/day) and Group V. HFD + HSE (500 mg/kg/day).

The animals were fed with HFD for 14 weeks to induce insulin resistance while normal control rats were fed with standard normal diet with normal drinking water. HFD contained 60% fructose, 20% casein, 0.3% methionine, choline bitartrate 0.2% 5% fat, 10% cellulose, 3.5% minerals and 1% vitamins mix. The composition of HFD followed the method of Guo and coworkers[9], and Suwannaph and coworkers[10]. After 14 weeks of HFD feeding, HSE or vehicle (distilled water) were intragastrically administered daily during the last 4 weeks (week 14^{th} - 18^{th}) of the study.

Oral glucose tolerance test (OGTT) assessments

Rats were fasted overnight (8-10 h) and blood samples were taken from a lateral tail vein to measure the fasting blood glucose using a glucometer (Roche Diagnostics Australia Pty. Ltd., Sydney, Australia). Then, the animals were orally administered with glucose at a dose of 2 g/kg body weight in order to determine glucose tolerance. The blood glucose concentration before glucose loading (fasting blood glucose or 0 min), at 30 and 120 min after glucose administration was investigated.

Hemodynamic and vascular responsiveness measurements

On the last day of the study, the animals were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). Body temperature was monitored using a rectal probe and maintained at 37 ± 2°C throughout the study using a heating pad. A femoral artery was identified, cleaned of a connective tissue, and cannulated with a polyethylene tube. Baseline values of systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) were continuously monitored for 20 min by a way of a pressure transducer and recorded using Acknowledge Data Acquisition software (Biopac Systems Inc., Santa Barbara, CA, USA).

After obtaining stable baseline data, a femoral vein was cannulated with a polyethylene tube. To test endothelial and smooth muscle cell function, a vascular responsiveness test was carried out by intravenous infusion of ACh, an endothelium-dependent vasodilator (3, 10, and 30 nmol/kg). Each dose of ACh was infused in stepwise concentration increases at 5-min intervals, with 5-min intervals between doses.

Assay of superoxide production.

Vascular $O_2^{\bullet -}$ production was measured using a lucigenin-enhanced chemiluminescence method[11] with some modifications[12].

Assay of nitrate and nitrite.

The concentration of plasma nitrate and nitrite (NOx) was measured using an enzymatic conversion method[13] with some modifications[12].

Western blot analysis

Protein eNOS expression levels were determined in aortic homogenates following a previously described western blot method[14], with some modifications[15].

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). The differences among treatment groups were analyzed by one-way analysis of variance (ANOVA). A p-value of less than 0.05 was considered statistically significant.

Results

Effects of HSE on oral glucose tolerance test

Significant increases in fasting blood glucose and impaired glucose tolerance were found in rats fed with HFD ($p < 0.05$). Treatment with HSE (200 and 500 mg/kg) for 4 weeks significantly reduced fasting blood glucose and recovered the impairment of glucose tolerance compared to those of rats fed with HFD without treatment ($p < 0.05$) (Figure 1). HSE had no effect on fasting blood glucose and oral glucose tolerance test in normal control rats.

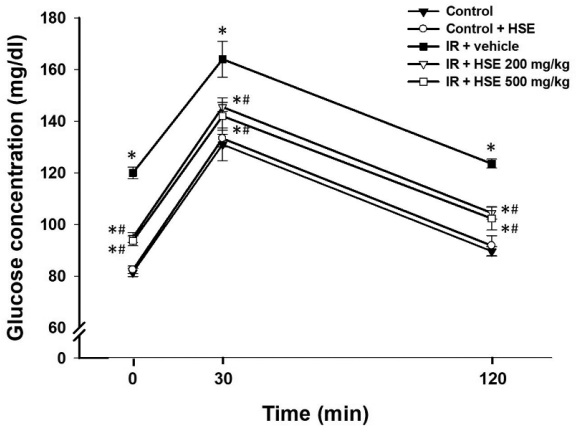

Figure 1 Effect of HSE on oral glucose tolerance test in HFD-fed rats. Results are expressed as mean ± SEM. *p<0.05 vs. control group, #p<0.05 vs. HFD group (n = 7/group).

Effects of HSE on mean arterial pressure

HFD induced mild hypertension, indicating by the significant increases in mean arterial pressure (MAP) in the HFD group comparing to control group (120.2 ± 3.1 mmHg vs. 93.6 ± 2.5 mmHg) ($p < 0.05$). However, treatment with HSE (200 and 500 mg/kg) for 4 weeks markedly reduced blood pressure in HFD group (109.9 ± 3.4 mmHg and 99.7 ± 3.2 mmHg) in a dose-dependent manner compared to untreated rats ($p < 0.05$). HSE had no effect on blood pressure in normal control rats (Figure 2).

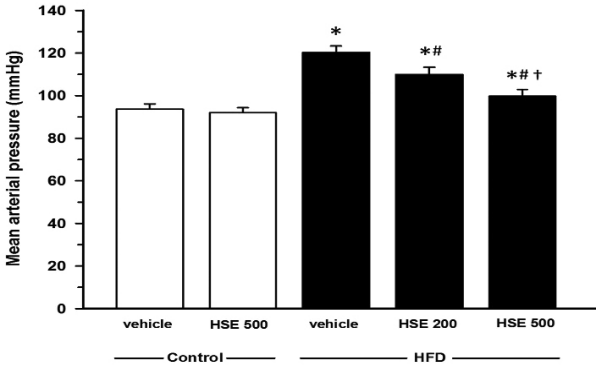

Figure 2 Effect of HSE on mean arterial pressure in HFD-fed rats. Results are expressed as mean ± SEM. *p<0.05 vs. control group, #p<0.05 vs. HFD group, †p<0.05 vs. HFD + HSE 200 mg/kg group (n = 7/group).

Effect of HSE on vascular responsiveness

Vasodilation responses to ACh were significantly blunted in HFD group when compared to that of normal control group; 30.0 ± 1.4% vs. 36.8 ± 1.1% at 3 nmol/kg, 42.7 ± 1.1% vs. 48.7 ± 1.1% at 10 nmol/kg, and 49.2 ± 0.5% vs. 57.6 ± 1.2% at 30 nmol/kg ($p < 0.05$) which may indicate that there was an endothelial dysfunction in rats fed with HFD. Oral supplementation of HES 200 mg/kg improved ACh-induced vasorelaxation in rats fed with HFD (54.1 ± 1.0% at 30 nmol/kg) ($p < 0.05$). Interestingly, treatment with HSE 500 mg/kg to rats fed with HFD markedly restored the vasodilation response to ACh at 3 nmol/kg (33.9 ± 1.0%), 10 nmol/kg (46.3 ± 1.7%) and (54.8 ± 1.4%) at 30 nmol/kg ($p < 0.05$) (Figure 3).

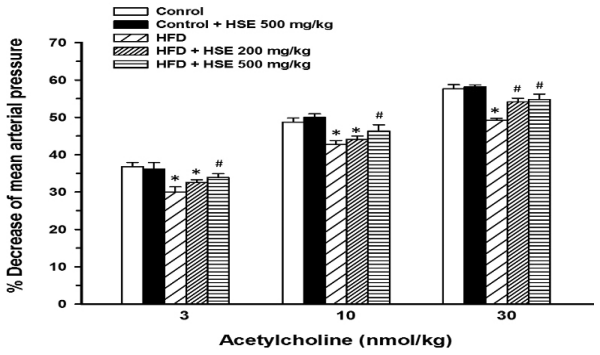

Figure 3 Effect of HSE on acetylcholine-induced vasodilation in HFD-fed rats. Results are expressed as mean ± SEM. *p<0.05 vs. control group, #p<0.05 vs. HFD group (n = 7/group).

Effect of HSE on plasma NOx levels and eNOS protein expression in aortic tissues

In rats fed with HFD, plasma NOx concentrations were significantly decreased (5.5 ± 0.3 μmol/L) compared with those in the normal control group (11.4 ± 0.8 μmol/L) ($p < 0.05$) (Figure 4A). Moreover, this reduction of plasma NOx was consistent with down regulation of eNOS protein expression in HFD-fed rats ($p < 0.05$) (Figure 4B). Oral supplementation with HSE (500 mg/kg) significantly improved the concentration of plasma NOx (10.3 ± 0.7 μmol/L; $p < 0.05$) and completely restored aortic eNOS protein expression in rats fed with HFD ($p < 0.05$).

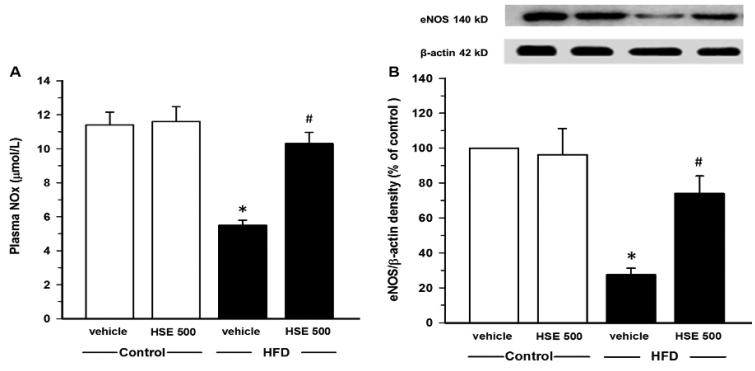

Figure 4 Effect of HSE on (A) plasma nitric oxide metabolites (NOx) and (B) aortic eNOS expression in HFD-fed rats. Results are expressed as mean ±SEM. *p<0.05 vs. control group, #p<0.05 vs. HFD group (plasma NOx n=7/group; eNOS expression n=4/group).

Effect of HSE on plasma $O_2^{\bullet-}$ production

Increased $O_2^{\bullet-}$ production in carotid arteries was found in the HFD group (110.4 ± 4.3 counts/min/mg dry weight) compared to that of the control group (56.8 ± 4.3 counts/min/mg dry weight) ($p < 0.05$) (Figure 5). HSE (200 and 500 mg/kg) supplementation markedly attenuated $O_2^{\bullet-}$ production in carotid arteries in HFD-fed rats (78.8 ± 3.8 and 66.3 ± 6.7 counts/min/mg dry weight, respectively) ($p < 0.05$).

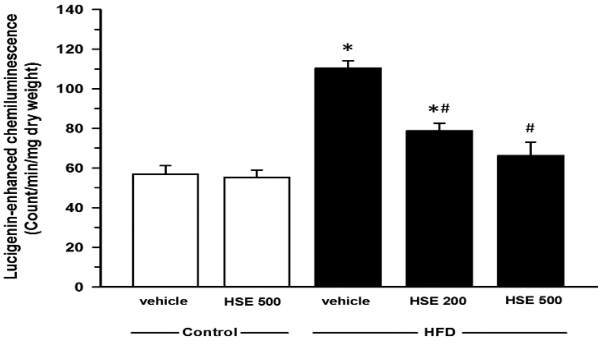

Figure 5 Effect of HSE on vascular superoxide production in HFD-fed rats. Results are expressed as mean ± SEM. *p<0.05 vs. control group, #p<0.05 vs. HFD group (n = 7/group).

Discussion

The main findings of this study were that HSE reduced hypertension as well as restored ACh-induced vasodilation with increasing plasma NOx and eNOS protein expression, and reducing oxidative stress markers in rats fed with HFD. Rats fed with HFD exhibited insulin resistance, characterized by impairment of fasting glucose and glucose tolerance. HSE supplementation reduced blood glucose and alleviated glucose tolerance in rats fed with HFD. The present results are in agreement with previous studies that demonstrated the anti-insulin resistance properties of *Hibiscus sabdariffa* polyphenolic extract and its effect on hypoglycemia in type 2 diabetic rats[4].

Cardiovascular complications induced by the HFD were characterized by endothelial, resulting in hypertension. These abnormality parameters were alleviated after HSE treatment. It was found that HFD-treated rats were blunted vascular response to ACh, indicated impairment of endothelium-dependent vasorelaxation. This confirms and extends previous reported the HFD-induced vascular endothelial dysfunction[16]. HSE reduced blood pressure in the present study involving the improvement of vascular function since a restoration of vascular responses to ACh was observed after HSE treatment. This result was supported by the previous studies that HSE improved endothelium-dependent vasorelaxation in insulin resistance rats[8] and spontaneously hypertensive rats[17].

Endothelial dysfunction found in rats fed with HFD was consistent with significantly decreasing plasma NOx concentration. NO is a gas molecule synthesized and derived from endothelial cells to mediate vasodilation[18]. Down regulation of eNOS protein expression reduces NO synthesis[3,19]. This study found the suppression of eNOS protein expression in aortic tissues in HFD-treated rats that were associated with low level of NO production. However, treatment of HFD-induced insulin resistance rats with HSE can raise plasma NOx concentration and completely restored eNOS protein expression. The one possible mechanism that HSE improved NO production, eNOS expression as well as vascular endothelial function may be link to its antihyperinsulinemia effect since, Potenza and coworkers reported that hyperinsulinemia induced endothelial dysfunction by decreasing eNOS expression and NO production[20]. Increasing in oxidative stress markers, such as lipid peroxidation and DNA oxidative damage have all been observed in HFD-treated rats[21]. In our experimental animals, we also found an increase in oxidative stress status, supporting by high levels of vascular $O_2^{\bullet-}$ production. We showed that oral supplementation with HSE was effective in decreasing vascular $O_2^{\bullet-}$ production in rats fed with HFD. Similarly, previous reported that the antioxidant activity of HSE is also due to its strong scavenging effect on reactive oxygen and free radicals[22], and inhibition of xanthine oxidase activity[23].

Conclusion

In summary, HSE reduced blood pressure and improved vascular endothelial function in a high-fructose diet-induced insulin resistance rats. This effect might involve with its ability to restore NO bioavailability, with concomitant upregulation of eNOS expression and reducing vascular $O_2^{\bullet-}$ production.

Acknowledgements

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