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ผลของลิโมนินต่อการทำงานและสัณฐานวิทยาของหลอดเลือดในหนูโรคอ้วน

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Effects of Limonin on Vascular Function and Morphology in a Rat Model of Obesity

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บทคัดย่อ

หลักการและวัตถุประสงค์: โรคอ้วนถูกรายงานว่ามีความเกี่ยวข้องกับความผิดปกติของหลอดเลือดและการเปลี่ยนแปลงทางสัณฐานวิทยา การศึกษานี้จึงมีวัตถุประสงค์เพื่อประเมินผลของลิโมนินต่อการทำงานของหลอดเลือดและสัณฐานวิทยาในหนูโรคอ้วนที่ถูกเหนี่ยวนำด้วยอาหารไขมันสูง
วิธีการศึกษา: หนูทดลอง Sprague-Dawley เพศผู้ ถูกแบ่งออกเป็น 3 กลุ่ม ได้แก่ กลุ่มควบคุมได้รับอาหารปกติและน้ำเปล่า กลุ่มอ้วนได้รับอาหารไขมันสูง ร่วมกับสารละลายฟรุกโตส 15 เปอร์เซ็นต์เป็นเวลา 16 สัปดาห์ และกลุ่มอ้วนที่ได้รับลิโมนินซึ่งได้รับอาหารไขมันสูงร่วมกับสารละลายฟรุกโตส 15 เปอร์เซ็นต์และได้รับลิโมนิน ขนาด 100 มิลลิกรัม/กิโลกรัม/วัน ในช่วง 4 สัปดาห์สุดท้าย เมื่อจบการศึกษา น้ำหนักตัว น้ำหนักไขมัน การตอบสนองของหลอดเลือด สัณฐานวิทยาของหลอดเลือด และตัวชี้วัดความเครียดออกซิเดชันถูกวัด
ผลการศึกษา: หนูโรคอ้วนมีการเพิ่มขึ้นของน้ำหนักตัวและไขมันในช่องท้อง ในขณะที่หนูโรคอ้วนที่ได้รับลิโมนินสามารถลดการเพิ่มขึ้นของน้ำหนักตัวและไขมันในช่องท้องได้ ($p<0.05$) ลิโมนินสามารถฟื้นฟูการทำงานของหลอดเลือดโดยลดการตอบสนองต่อการกระตุ้นด้วยไฟฟ้าในหลอดเลือดมีเซนเทอริกและลดความบกพร่องของการตอบสนองต่อสารอะเซทิลโคลีนในหลอดเลือดเอออร์ต้าของหนูโรคอ้วน ($p<0.05$) ความผิดปกติของลักษณะทางสัณฐานวิทยาของหลอดเลือดกลับสู่ค่าปกติในหนูโรคอ้วนที่ได้รับสารลิโมนิน นอกจากนี้การผลิตซูเปอร์ออกไซด์ ของหลอดเลือดที่เพิ่มขึ้น ระดับเมแทบอไลต์ของไนตริกออกไซด์ในพลาสมาที่ลดลงในหนูโรคอ้วนบรรเทาลงด้วยการให้ลิโมนิน ($p<0.05$)
สรุป: ลิโมนินลดการทำงานและสัณฐานวิทยาที่ผิดปกติของหลอดเลือดในหนูโรคอ้วน กลไกที่เป็นไปได้อาจเกี่ยวข้องกับคุณสมบัติต้านโรคอ้วนและต้านอนุมูลอิสระ

คำสำคัญ: ลิโมนิน, การทำงานและสัณฐานวิทยาของหลอดเลือด, โรคอ้วน, ภาวะเครียดออกซิเดชั่น

Abstract

Background and Objectives: Obesity has been reported to be associated with vascular dysfunction and morphology changes. This study aimed to evaluate the effect of limonin on vascular function and morphology in high fat (HF) diet-induced obesity in rats.
Methods: Male Sprague-Dawley rats were divided into 3 groups; control group fed with normal diet and tap water; obese group fed with a HF-diet together with 15% fructose solution for 16 weeks and obese+LM100 group fed with a HF-diet plus 15% fructose solution, and limonin (100 mg/kg) for the last 4 weeks. At the end of the study, body weight (BW), retroperitoneal fat weight (RFW), vascular function, vascular morphology and oxidative stress markers were determined.
Results: Obese rats had increases in BW and RFW while treatment with limonin can attenuate the high BW and RFW ($p<0.05$). Limonin improved vascular function by reducing the enhancement of contractile response to electrical filed stimulation in the mesenteric vascular beds and attenuated the impairment of vasorelaxation response to acetylcholine in aortic rings isolated from obese rats ($p<0.05$). Aortic hypertrophy, indicated by increases in cross-sectional area, aortic wall thickness and wall/lumen ratio, was normalized in obese rats after limonin treatment. Moreover, increased vascular superoxide production, and decreased plasma nitric oxide metabolites were also observed in obese rats and these were restored by limonin treatment ($p<0.05$).
Conclusion: Limonin alleviated vascular dysfunction and hypertrophy in obese rats. The possible mechanism might associate with its anti-obesity and antioxidant properties.

Keywords: Limonin, vascular function and morphology, obesity, oxidative stress

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Introduction

Obesity has been known as a chronic medical condition that is defined as an excess of body weight and fat accumulation[1]. Obesity is a major risk factor for cardiovascular diseases, hypertension, stroke and type 2 diabetes mellitus[2]. It is well known that excessive calorie intake is a major cause of obesity in humans[3]. In a rat model of high-fat diet (HF-diet)-induced obese rats, it has been shown to have an elevation of body weight, retroperitoneal fat, and vascular dysfunction[4,5]. In addition, an impairment of endothelium-dependent vasorelaxation has been observed in vascular tissue from obese rats[6]. The augmentation of contractile response to sympathetic nerve stimulation was also reported in rats received excessive caloric diet[7]. These vascular alterations in obese rats have been linked to a variety of causes, including decreased nitric oxide (NO) bioavailability, and increased oxidative stress[7,8]. Additionally, increases in oxidative stress markers have been reported to contribute to the development of vascular remodeling in an animal model of HF-diet rats[9].

Limonin is a triterpenoid aglycone and the most bioactive phytochemical component of limonoids. It is found predominantly in citrus fruits, particularly citrus seeds[10]. Limonin exhibited anti-oxidant and anti-inflammatory effects in non-alcoholic fatty liver disease in zebrafish[11]. The cardio-protective effects of limonin in doxorubicin-mediated cardiotoxicity of myocardial cell line H9C2 were demonstrated which was related with its ant-oxidant effects[12]. This study is designed to investigate the effect of limonin on obesity, vascular function and morphology, oxidative stress status in HF-diet-induced obese rats.

Materials and Methods

Chemicals

Limonin was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Norepinephrine (NE) was purchased from Sigma-Aldrich Corp (St Louis, MO, USA). Phenylephrine (PE) was purchased from Sigma-Aldrich Corp (St Louis, MO, USA). Acetylcholine (ACh) and sodium nitroprusside (SNP) were purchased from Fluka Chemika (Buchs, Switzerland).

Animals

Adult male Sprague-Dawley rats (220-270 g) were purchased from Nomura Siam International Co., Ltd., Bangkok, Thailand. All animals were given standard food and water *ad libitum* for 6 weeks. Rats were maintained under a standard system at 23 ± 2 °C with a 12-h light / 12-h dark cycle at the Northeast Laboratory Animal Center, Khon Kaen University, Thailand. All animal procedures following the ethical guideline for work with animals were controlled and approved by the Institutional Animal Care and Use Committee of Khon Kaen University (Ethics No IACUC-KKU-51/64).

Experimental protocols

Rats were divided into 3 groups (n=5/group) including, Control group: rats received a stand chow diet+ vehicle, Obese group: rats received a HF-diet + vehicle, Obese-treated group: rats received a HF-diet + limonin at a dose 100 mg/kg/day. The control rats were given tap water while obese rats were given 15% fructose in drinking water for 16 weeks. Limonin or propylene glycol (vehicle) was orally administered for the last 4 weeks.

Body weight and fat weight measurements

Rat body weight was measured once a week. The retroperitoneal fat was collected and weighed at the end of experiment after euthanasia.

Sample collections

At the end of experiments, all animals were intraperitoneally injected with thiopental sodium (70 mg/kg) and blood samples were collected via abdominal aorta for measuring oxidative stress parameters. The mesenteric vascular bed and thoracic aorta were collected for analyzing vascular function and morphology.

Vascular responses to vasoactive agents and electrical field stimulation

The mesenteric vascular bed was carefully isolated and then placed on a stainless-steel grid (7×5 cm) in a humid chamber. The preparations were perfused with physiological Krebs' solution continually gassed with a 95% O_2 and 5% CO_2 gas mixture at a constant flow rate of 5 mL/min, using a peristaltic pump (07534-04, Cole-Palmer Instrument, Illinois, USA). Contractile responses to electrical field stimulation (EFS) (5-40 Hz, 90 V, 1 ms, for 30 s at 5 min intervals) and bolus injection of NE (0.15-15 nmol) were performed. The contractile response was detected as change in mean perfusion pressure (mmHg) using a pressure transducer and data recoded via the BIOPAC system (BIOPAC system Inc., California, USA).

To assess vasoactive performance of a conduit artery, the thoracic aorta was rapidly removed and cut into rings 2-3 mm in length, and the rings were mounted in 15 mL baths containing Krebs' solution.

The aortic rings were raised tone with PE, a selective $_1$ receptor agonist, (10 μM), after that Ach and SNP (0.01-3μM) was added into the organ bath to evaluate vascular relaxation.

Histological examination of thoracic aorta

The thoracic aorta was fixed with 4% paraformaldehyde, embedded in a paraffin block, and cut in to 5µm thickness. Each section was stained with hematoxylin and eosin (HAE)stain. Thereafter, sections were observed under the Digital sight DS-2MV light microscope (Nikon, Tokyo, Japan) at 4x and 20x objective lens. Images were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA)

Oxidative stress markers measurements

Assay of superoxide ($O_2^{\bullet-}$) production in thoracic aorta

Vascular $O_2^{\bullet-}$ production was detected in the thoracic aorta using lucigenin-enhanced chemiluminescence method as previously described by Lu et al. in 1996[13]. The aortas were isolated, cut and incubated in the tube filled with oxygenated Krebs-KCl solution (37°C) for 30 min. Thereafter, the sample tubes with lucigenin were placed in a luminometer (Turner Biosystems, CA, USA). The luminometer detections were counted every 30 seconds for 5 min. The production of $O_2^{\bullet-}$ was expressed as relative light unit counts per min per dried weight of the vessel.

Assay of plasma nitric oxide metabolites (NOx) level

Plasma NOx concentration were measured using an enzymatic conversion method as previously described by Verdon et al. in 1995[14] with minor modifications[15]. Briefly, the plasma was deproteinized and the supernatant was mixed with NADPH, G-6-P, 1 G-6-PD and nitrate reductase before incubated at 30 °C for 30 min. After that, the mixer was reacted with a Griess solution for 15 min. The absorbance of the samples was detected at wavelength 540 nm using a microplate reader (Tecan GmbH., Groding Australia). A standard curve was established with a set of serial dilution of $NaNO_2$[16].

Statistical analysis

The results were expressed as the mean ± S.E.M. One-way ANOVA followed by Tukey post-hoc test for comparison between groups was used to test statistical analysis. A p-value of less than 0.05 was considered statistically significant.

Results

Effect of limonin on body weight and retroperitoneal fat weight/body weight

Body weight and retroperitoneal fat weight/body weight in week 16 were significantly increased in the obese group (949.20 ± 46.96 g and 72.34 ± 7.01 mg/g) compared to the control group (683.20 ± 9.70 g and 36.21 ± 2.58 mg/g) ($p<0.05$). Treatment with limonin (100 mg/kg) significantly decreased body weight and retroperitoneal fat weight/body weight (768.80 ± 28.20 g and 52.59 ± 0.99 mg/g) compared to the untreated group ($p<0.05$) (Figure 1).

Effect of limonin on vascular responses to electrical field and vasoactive agents

Vascular contractile responses to EFS in an isolated mesenteric vasculature of obese rats were significantly increased compared to the control rats ($p< 0.05$). Treatment with limonin (100 mg/kg) significantly decreased vasoconstriction responses to EFS compared to the untreated rats ($p< 0.05$) (Figure 2A). However, the response to exogenous NE did not differ among the groups (Figure 2B).

Effect of limonin on endothelial-dependent vasodilation in aortic rings

Obese group showed an impairment of vasorelaxation responses to ACh in aortic rings compared to the control group ($p< 0.05$). Treatment with limonin significantly improved the response to ACh compared to the untreated group ($p< 0.05$) (Figure 3A). However, vasorelaxation responses to SNP were not difference among groups (Figure 3B).

Effect of limonin on thoracic aorta morphology

Morphological changes in thoracic aortas were observed in obese rats induced by a HF-diet (Figure 4A). Significant increases in the cross-sectional areas (CSA), aortic wall thickness and wall/lumen ratio were observed in the obese group ($p < 0.05$). Limonin (100 mg/kg) treatments significantly reduced vascular hypertrophy in obese rats compared to the untreated group ($p < 0.05$) (Figure 4B, C and E). There was no significantly difference in luminal area among the groups (Figure 4D).

Effect of limonin on oxidative markers

Vascular $O_2^{\bullet-}$ production was significantly elevated in the obese group compared with the control group ($p<0.05$). Treated with limonin (100 mg/kg) significantly decreased $O_2^{\bullet-}$ production compared to the untreated group ($p<0.05$) (Figure 5A). The levels of plasma NOx in obese rats were significantly lower than those of the control rats ($p<0.05$). Limonin (100 mg/kg) treatments significantly restored plasma NOx compared to the untreated group ($p<0.05$) (Figure 5B).

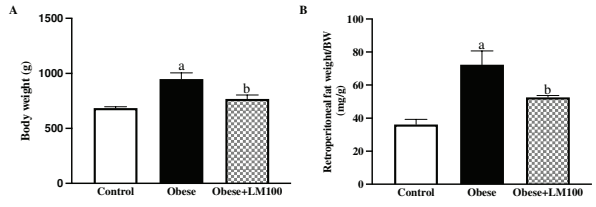

Figure 1 Effects of limonin on rat body weight (A) and retroperitoneal fat weight/body weight (B). Data are presented as the mean ± S.E.M. (n = 5).[a] p<0.05 significant difference with control group,[b] p<0.05 significant difference with obese group, LM100=limonin (100 mg/kg).

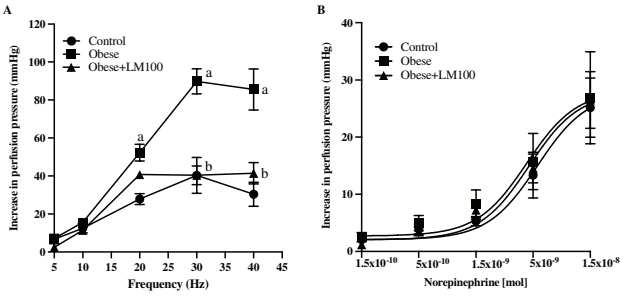

Figure 2 Effects of limonin on contractile responses to EFS (5-40 Hz, 90 V, 1 ms, for 30 s at 5 min intervals) (A) and bolus injection of norepinephrine (0.15-15 nmol) under basal tone conditions (B). Data are presented as the mean ± S.E.M. (n = 5).[a] p<0.05 significant difference with control group,[b] p<0.05 significant difference with obese group, LM100=limonin (100 mg/kg).

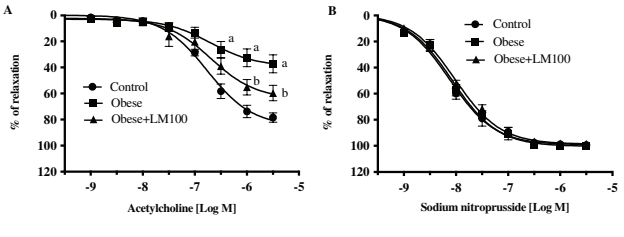

Figure 3 Effects of limonin on vasorelaxation induced by acetylcholine (0.01-3μM) (A) and sodium nitroprusside (0.01-3μM) (B) in aortic rings under phenylephrine-raised tone condition (10 μM). Data are presented as the mean ± S.E.M. (n = 5).[a] p<0.05 significant difference with control group,[b] p<0.05 significant difference with obese group, LM100=limonin (100 mg/kg).

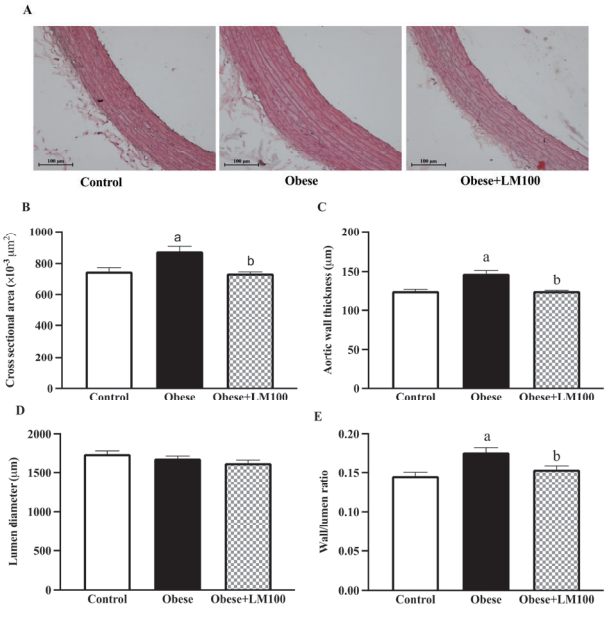

Figure 4 Effects of limonin on thoracic-aorta morphology in HF-diet-induced obese rats. Representative images of aortic morphology sections stained with H&E (magnification is X20) (A). Effects of limonin on cross-sectional areas (B), aortic wall thickness (C), Lumen diameter (D), Wall/lumen ratio (E). Data are presented as the mean ± S.E.M. (n=5).[a] p<0.05 significant difference with control group,[b] p<0.05 significant difference with obese group, LM100=limonin (100 mg/kg).

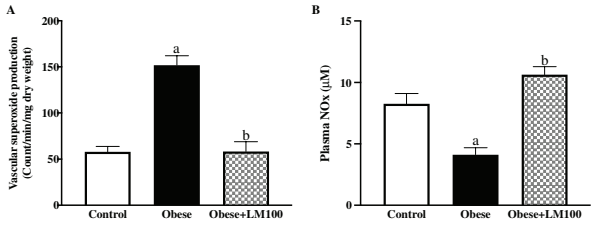

Figure 5 Effects of limonin on vascular superoxide production (A) and plasma nitrate/nitrite (B). Data are presented as the mean ± S.E.M. (n=5).[a] p<0.05 significant difference with control group,[b] p<0.05 significant difference with obese group, LM100=limonin (100 mg/kg).

Discussion

This study found the beneficial effects of limonin on vascular dysfunction and hypertrophy induced by a HF-diet in rats. The results showed that rats fed with a HF-diet has increased body weight, retroperitoneal fat weight/body weight, augmented vascular contractile responses to EFS and decreased vascular responses to ACh and these were alleviated after treatment with limonin. Vascular hypertrophy was found in obese rats

that were supported by increases in CSA, aortic wall thickness and wall/lumen ratio. Limonin attenuated high levels of vascular $O_2^{\bullet-}$ production, and low level of plasma NOx in obese rats.

This study showed that rats that received an HF-diet plus 15% fructose in drinking water had increased body weight as well as retroperitoneal fat weight/body weight. It is well recognized that an imbalance between energy intake and energy expenditure causes obesity. Excessive-calorie consumption was noted to be the main factor contributing to the pathogenesis of obesity since it promotes lipid accumulation in adipocytes resulting in increased adipocyte number and adipocyte sizes[17]. In addition, high-calorie diet especially a HF-diet has been used in several animal studies to induce obesity and its complications[4,18]. For instance, Bhandarkar and coworkers reported that feeding the rats with a high-carbohydrate, high-fat diet for 16 weeks caused increases in body weight, waist-circumference and retroperitoneal fat weight in metabolic syndrome rats[4].

Oxidative stress play a crucial role in the pathogenesis of HF-diet-induced obesity, vascular dysfunction, and remodeling in rats[19]. Recent studies have revealed that obesity is associated with a high level of angiotensin II which promotes oxidative stress in vasculature through several mechanisms, including activation of NOx, formation of $O_2^{\bullet-}$ radicals and production of H_2O_2[20]. Increased body weight with excess fat accumulation leads to excessive production of $O_2^{\bullet-}$, which may react with nitric oxide to form peroxynitrite, resulting in decreased nitric oxide availability[21]. Reactive oxygen species also influences vascular remodeling by increasing collagen and other extracellular matrix protein deposition in the blood vessel[22]. In the present study, a high level of oxidative stress was noted since the production of $O_2^{\bullet-}$ radicals in aortic tissue was increased in obese rats. Moreover, endothelial dysfunction was observed in the thoracic aorta. The vasorelaxation response to ACh was reduced whereas the response to SNP was not changed. Furthermore, vasoconstriction responses to EFS were enhanced in obese rats but the vasoconstriction response to an exogenous NE did not differ among groups, indicating an over-activation of sympathetic nerve in the mesenteric vascular bed of the obese rats. Similar observations have been reported that impairment of endothelial function together with sympathetic nerve overactivity were associated with the reduction of nitric oxide bioavailability and the increase in oxidative stress production in metabolic syndrome rats fed with high-carbohydrate, high-fat diet[7]. Subsequently, another study reported that HF-diet induced obese mice had diminished reduction of ACh-induced vasodilation in aortic rings via reducing nitric oxide production[23]. It is well documented that rats fed with HF-diet expressed obesity, vascular endothelial dysfunction, and alterations of vascular morphology[9]. Furthermore, an elevation of vascular $O_2^{\bullet-}$ production and a reduction of plasma NOx concentration have been reported in HF-diet rats[9]. Interestingly, the present study found that supplementation with limonin alleviated obesity, vascular dysfunction, and vascular morphological changes in obese rats. Previous study has been reported that limonin exhibited antioxidant property[10,24] by its free radical scavenging activity and inhibiting the formation of $O_2^{\bullet-}$ radicals[25]. One possible mechanism underlying of the therapeutic effect of limonin against obesity and vascular alterations in HF-diet-fed rats might be attributed to its anti-oxidative property.

Conclusion

In summary, the present study demonstrates that limonin alleviated vascular dysfunction and structural changes in obese rats via decreasing vascular $O_2^{\bullet-}$ production and raising nitric oxide bioavailability.

Acknowledgements

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