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D-chiro-inositol enriched *Fagopyrum tataricum* (L.) Gaench extract alleviates mitochondrial malfunction and inhibits ER stress/JNK associated inflammation in the endothelium

Bobo Zhang[a], Caifeng Gao[a], Yunlong Li[b], Min Wang[a,*]

[a] College of Food Science and Engineering, Northwest A&F University, Yangling 712100, PR China
[b] Institute of agricultural products processing, Shanxi Academy of Agriculture Sciences, Taiyuan 030031, PR China

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ABSTRACT

Ethnopharmacological relevance: Tartary buckwheat is a food medicine dual-use crop with healing effects on cardiovascular diseases and type2 diabetes. It has been proposed that endothelial dysfunction is the initial lesion in these diseases and it's associated with mitochondrial dysfunction, endoplasmic reticulum (ER) stress and inflammation. D-chiro-inositol (DCI) is a bioactive compound of Tartary buckwheat and is always deficit in type2 diabetes. However, it remains unknown whether DCI-enriched Tartary buckwheat extract can ameliorate mitochondrial dysfunction, ER stress and inflammation in the endothelium.

Material and methods: Endothelial cells were treated with palmitic acid (PA) and mice were fed with high fat diet (HFD). The effects of DCI-enriched Tartary buckwheat bran extract (TBBE) on superoxide anion generation, dynamin-related protein 1 (Drp1), mitofusin2 (Mfn2), inositol-requiring enzyme-1α (IRE1α) and Jun n-terminal kinase (JNK) activation and inflammation in the endothelium against lipotoxicity were investigated.

Results: In endothelial cells, TBBE significantly inhibited oxidative stress. Meanwhile, in HFD-fed mice and PA-induced cells, TBBE regulated Drp1 phosphorylation and inhibited its activation, implying the protective effect of TBBE on mitochondrial morphology. As a result, TBBE protected mitochondrial function. Additionally, TBBE inhibited ER stress and reduced the production of IL-6 and VCAM-1, associated with JNK pathway, thereby inhibiting the caspase-3 activation *in vivo* and *in vitro*.

Conclusions: Taken together, this study indicated the beneficial role of TBBE in endothelial inflammation, with emphasis on mitochondrial dysfunction, ER stress and JNK activation.

1. Introduction

Endothelial dysfunction is a main feature of cardiovascular diseases, including atherosclerosis, thrombosis and hypertension (Kratz et al., 2013; Mazzoccoli et al., 2012; Gkaliagkousi et al., 2015). Lipid disorder, especially high level of circulating free fatty acids (FFAs) has been shown to be responsible for endothelial dysfunction (Kim et al., 2006), wherein the oxidative stress is considered to be the proximal reason (Kim and Byzova, 2014; Han and Kaufman, 2016). Mitochondria are the major sites for reactive oxygen species (ROS) generation. Meanwhile, mitochondrial integrity is crucial for the suppression of oxidative stress (Li et al., 2016). Mitochondria are tubular organelles whose morphology is regulated by dynamic homeostasis of fusion and fission. Dynamin-related protein 1 (Drp1) is a key factor in the regulation of this process. The phosphorylation at Ser616 increases Drp1 activity, leading to mitochondrial fission, whereas the phosphorylation of Drp1 at Ser637 prevents mitochondrial fission (Kageyama et al., 2011) and ameliorates mitochondrial malfunction in endothelial cells (Li et al., 2016).

It is well known that endoplasmic reticulum (ER) tubules mark sites of mitochondrial fission, indicating the functional link between ER and mitochondria (Friedman et al., 2011). The ER serves for the synthesis of lipid, the folding of protein, and also mediates intracellular signal transduction (Lin et al., 2008). In response to stress, the accumulation of unfolded protein response (UPR) leads to trans-membrane sensors activation, including inositol-requiring enzyme-1α (IRE1α). Although

Abbreviations: CMC-Na, carboxymethylcellulose sodium; DCI, D-chiro-inositol; DHE, dihydroethidium; Drp1, dynamin-related protein 1; ER, endoplasmic reticulum; FFAs, free fatty acids; HFD, high fat diet; IRE-1α, inositol-requiring enzyme-1α; JNK, Jun n-terminal kinase; Mdivi-1, mitochondrial division inhibitor-1; Met metformin, Mfn2 mitofusin2; PA, palmitate acid; RAECs, rat aortic primary endothelial cells; RIPA, Radio Immunoprecipitation Assay buffer; ROS, reactive oxygen species; TBBE, tartary buckwheat bran extract; UPR, unfolded protein response; VCAM-1, vascular cell adhesion molecule-1; Δψm, mitochondria membrane potential

* Corresponding author.
E-mail addresses: wangmin20050606@163.com, wangmin20110606@163.com (M. Wang).

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UPR is an adaptive process to regulate homeostasis of cell, the accumulation of UPR induces ER stress (Lin et al., 2008). It has been reported that ER stress induces inflammatory response (Mills et al., 2017), even plays an important role in the pathogenesis of cardiovascular diseases (Kusaczuk and Cechowska-Pasko, 2013), and the inhibition of ER stress restores the loss of endothelium-dependent relaxation (Matsumoto et al., 2016). Meanwhile, Jun n-terminal kinase (JNK) links mitochondrial malfunction and ER stress to inflammation and apoptosis (Joo et al., 2016; Yang et al., 2014). In response to stress, IRE1α activation and mitochondrial dysfunction induce the activation of JNK (Zhang and Kaufman, 2008; Kaneto et al., 2005; Li and Yu, 2013), which promotes pro-inflammatory cytokine maturation. Thus, these events imply the involvement of mitochondrial malfunction and ER stress/JNK activation in endothelial dysfunction.

As a food medicine dual-use plant, Tartary buckwheat (*Fagopyrum tataricum* (L.) Gaench) has healing effects over chronic diseases (Zhang et al., 2012), including diabetes (Qiu et al., 2016) and hypertension (Cheng et al., 2016), in which endothelial dysfunction is an early feature. In the present study, D-chiro inositol, an excellent bioactive compound from Tartary buckwheat (Zhang et al., 2012), has been used to prevent endothelial dysfunction in diabetic rats (Nascimento et al., 2006). However, it remains unclear whether DCI-enriched Tartary buckwheat extract can inhibit JNK-associated inflammation in the endothelium by ameliorating mitochondrial dysfunction and ER stress. In this paper, the potential effects of DCI-enriched Tartary buckwheat extract on improving endothelial function against lipotoxicity were investigated.

2. Materials and methods

2.1. Materials

DCI-enriched Tartary buckwheat (*F. Tataricum* (L.) Gaench, voucher specimen num. #20100816 (Hu et al., 2012)) bran extract (TBBE) was stored in −80 °C and gifted from Shanxi Academy of Agriculture Sciences. TBBE was prepared by soaking the dried powder of Tartary buckwheat bran with 60% ethanol aqueous solution. The supernatant was filtered, concentrated, steamed and then refined with the activated carbons and strong acidic styrene cation ion-exchange resin and strong alkaline styrene anion ion-exchange resin, in turn. The extracts were further filtered and concentrated. HPLC analysis of TBBE revealed three bioactive compounds, namely: D-chiro inositol, myo-inositol and rutin.

For animal experiments, TBBE and metformin (Met) were suspended in 0.3% of carboxymethylcellulose sodium (CMC-Na). For cell experiments, palmitate acid (PA) was prepared to 200 nmol/L by ethanol and diluted to working concentration (100 μmol/L); TBBE and mitochondrial division inhibitor-1 (Mdivi-1) were dissolved in DMSO (working concentration of DMSO was under 0.1%). The following antibodies were from the cited commercial sources: anti-cleaved caspase-3, anti-phospho-Drp1 (Ser616), anti-JNK and anti-phospho-JNK (Thr183/Tyr185), Cell Signaling Technology (Beverly, MA, USA); anti-Drp1, anti-GAPDH, goat anti-rabbit IgG (H + L) HRP, Bioworld Technology (St. Paul, MN, USA); anti-phospho-IRE1α (S724), anti-IRE1α, anti-phospho-Drp1(Ser637), anti-Mitofusin 2, anti-prohibitin, Abcam (Cambridge, MA, USA).

2.2. Cell preparation and culture

Rat aortic primary endothelial cells (RAECs) were obtained from rat thoracic aortas as described (Magid et al., 2009). RAECs were cultured in DMEM with 20% of FBS, penicillin G (100 U/mL) and streptomycin (100 μg/mL), in a humidified atmosphere of 5% CO_2 at 37 °C. The cells used for the experiment were from the passages 3–6.

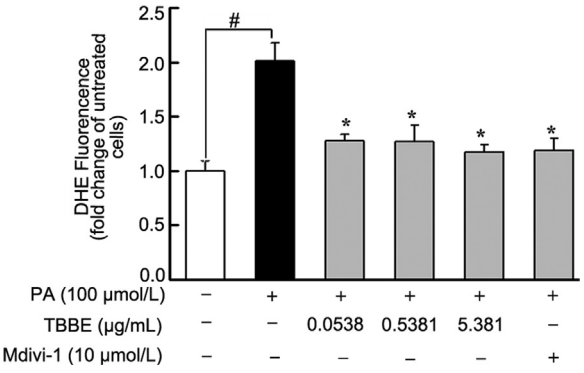

Fig. 1. TBBE reduced superoxide production in RAECs. The effect of TBBE on superoxide generation was assayed by using DHE labeling (n = 6). Date are means ± SD. *$P < 0.05$: TBBE and Mdivi-1 groups vs PA group; #$P < 0.05$: untreated group vs PA group.

2.3. Animals and experimental groups

The animals experiments were carried out at the Department of Complex Prescription of TCM, Institute of China Pharmaceutical University, Nanjing, China. Sprague-Dawley (SD) rats (200–250 g) and Institute of Cancer Research (ICR) male mice (20–22 g, 6 weeks) were cared and treated with the guidelines and approved by Animal Ethics Committee of China Pharmaceutical University in Nanjing, China (SCXK 2012-0004).

The mice had free access to water and food in cages (five per cage) and were randomly divided into five groups (n = 10). Normal group (NFD): the mice were receiving standard diet with oral administrated of 0.3%CMC-Na for 14 days. High fat diet (HFD) group: the mice were receiving high fat diet with oral administrated of 0.3%CMC-Na. HFD consists of chocolate (10%), cholesterol (10%), lard (1%), yolk (0.2%), and a standard diet (78.8%). TBBE groups: (1) the mice were receiving HFD and oral administrated of TBBE (74.67 mg/kg body weight), (2) the mice were receiving HFD and oral administrated of TBBE (149.3 mg/kg body weight). Met group: the mice were receiving HFD and oral administrated of Met (200 mg/kg body weight).

2.4. Superoxide anion production analysis

In order to assay superoxide anion generation, RAECs were co-treated with TBBE at concentration ranging from 0.05381, 0.5381, 5.381 μg/mL and 10 μmol/L of Mdivi-1, then were treated with 100 μmol/L of PA for 24 h. Superoxide anion was determined by dihydroethidium (DHE) staining probe (Beyotime Institute of Biotechnology, Shanghai, China).

2.5. Western blot analysis

In order to assay protein, cells were treated with 0.05381, 0.5381, 5.381 μg/mL of TBBE and 10 μmol/L of Mdivi-1, then were treated with PA for 24 h, lysed in cold Radio Immunoprecipitation Assay buffer (RIPA) (with 1 mmol/L of PMSF), and incubated with ice-water for 45 min. Protein was obtained by centrifugation at 12,000*g* for 20 min at 4 °C, and quantified by using Bicinchoninic Acid Protein Assay Kit (Biosky Biotechnology Corporation, Nanjing, China). Equal amounts of protein were separated by SDS-PAGE, transferred to polyvinylidene fluoride membranes. The membranes were incubated with specific antibodies at 4 °C overnight, and then with secondary antibody for 37 °C for 2 h. ECL Western Blotting Detection System was used to assay antibody-antigen complexes.

2.6. Mitochondria membrane potential (Δψm) analysis

In order to assay Δψm, RAECs were pre-treated with TBBE at