

Danggui Buxue Tang, a Chinese Herbal Decoction, Induces Nitric Oxide Production in Cultured Endothelial Cells: Probing Calycosin as a Key Player by Chemical Knock-Out Approach

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Abstract

Danggui Buxue Tang (DBT) is a Chinese herbal decoction prescribed for woman menopausal symptoms. The make-up of DBT is Astragali Radix (AR) and Angelicae Sinensis Radix (ASR) at a weight ratio of 5:1. In cultured endothelial cells, application of DBT induced the production of nitric oxide (NO), the phosphorylations of eNOS and Akt, and the mobilization of intracellular calcium. The chemicals within DBT responsible for the effects in endothelial cells however have never been addressed. Here, we developed a chemical knock-out method in a herbal mixture as to determine the role of individual ingredient(s). Calycosin is the most abundant flavonoid in DBT herbal decoction. By HPLC separation, calycosin was removed at over 97% from DBT decoction, which was named as DBT_{Δcal}. Comparing to authentic DBT, the induction of NO production as well as its signaling responses, triggered by DBT_{Δcal}, in cultured endothelial cells were markedly reduced. In contrast, calycosin alone showed no response in the cultures. Our finding showed a synergistic role of calycosin in the herbal mixture.

Keywords: Danggui buxue tang; Calycosin; Flavonoid; Chinese medicine

Introduction

Traditional Chinese Medicine (TCM) has been utilized for thousands of years, along with a long history; TCM has become a special system to cure illness. The theory of TCM was based on holistic idea of universe outline in spiritual insights of Daoism, and which had created a highly sophisticated set of practices designed to cure illness and balance the whole body [1]. The concept of “Yin-Yang and Five-Elements” are the basic principle of TCM. The “Five-Element” refers to “Wood, Fire, Metal, Earth and Water”, and which is a holistic view of the universe and human body [2]. Herbal formulated decoction is a common usage of TCM, which composes of at least two herbs. Usually, the action of single herb is rather limited, and which may produce side effects or even toxicity. The above mentioned disadvantages could be compensated by combining several herbs together. The herbal formula should be prepared in a special methodology, and the role of individual herb could be classified in accord to the theory of Jun (Master), Chen (Minister), Zuo (Assistant) and Shi (Servant) [3]. Endothelium, formed by endothelial cell, was a thin layer of cells lining the interior surface of blood vessels forming an interface between circulating blood in lumen and vessel wall. The endothelial dysfunction in many clinical situations led to atherosclerosis and coronary heart diseases [4,5]. For example, the low levels of nitric oxide (NO) and endothelium-derived relaxation factors, released by endothelial cells, were found in atherosclerosis [6,7]. NO is an important mediator for vasodilation in blood vessels [5,8,9], which benefits vessel homeostasis by inhibiting smooth muscle contraction, growth and platelet aggregation [5,8,9].

Danggui Buxue Tang (DBT), a Chinese herbal decoction, contains two herbs, Astragali Radix (AR) and Angelicae Sinensis Radix (ASR) at a ratio of 5:1 [10-12]. In clinical application, DBT is being used to treat woman menopausal illness and cardiovascular diseases [5]. Here, AR serves as “Master” herb, while ASR serves as “Minister” herb in DBT decoction. AR, one of the most popular herbs found in TCM formula, contains high amount of phytoestrogen that shares similarity with the functional group of 17β-estradiol. Clinically, phytoestrogen has been applied for myocardial dysfunctions and mitigating menopausal illnesses [13]. Because of complexity of herbal decoctions, the contribution of one chemical within a decoction is hard to be identified [14]. Calycosin is a major flavonoid in AR, therefore as in DBT [10], which is also considered as a bioactive chemical [3,15,16]. Here, we would like to reveal the contribution of one chemical within DBT decoction, as well as to clarify its synergistic roles in orchestrating biochemical actions within the decoction. By using semi-preparative HPLC in specific chemical depletion strategy, a calycosin-depleted DBT, named as DBT_{Δcal} was generated. The relationship of calycosin and DBT decoction in terms of NO production in endothelial cells was evaluated.

Materials and Methods

Plant materials and preparation of herbal decoctions

The roots of three-year-old *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao (AR) from Shanxi Province and two-year-old *Angelicae sinensis* (Oliv.) Diel roots (ASR) from Gansu Province were harvested in 2013. The authentication of plant materials was identified morphologically by Dr. Tina Dong at Hong Kong University of Science and Technology (HKUST). The voucher

specimens were deposited in the Centre for Chinese Medicine R&D at the university. Ferulic acid was purchased from Sigma (St. Louis, MO); calycosin, formononetin, and Z-ligustilide were purchased from TLCM (HKUST, Hong Kong China). The purities of these marker chemicals, confirmed by high-performance liquid chromatography (HPLC), were higher than 98.0%. Analytical- and HPLC-grade reagents were from Merck (Darmstadt, Germany).

Preparation of DBT decoctions

Thirty grams of AR and 6 grams of ASR were weighed to prepare DBT: the mixtures were boiled in 8 volumes of water (V/W) for 2 hours, repeated twice. This method of preparation followed the ancient recipe that had been shown to have the best extracting efficiency [17]. The extract was lyophilized into powder and re-suspended in water at a final concentration of 100 mg/mL. Depletion was conducted on an Agilent 1200 series system (Agilent, Waldbronn, Germany), equipped with a degasser, a binary pump, an auto-sampler, and a thermo-stated column compartment and Dikma, Diamonsil, C18 column (10.0 mm × 250, 5 μm) to create the calycosin-depleted DBT (DBT_{Δcal}). The specific depletion method was described previously [14].

Fingerprints of DBT decoctions

For LC-MS fingerprint analysis, an Agilent 1200 series system (Agilent, Waldbronn, Germany), equipped with a degasser, a binary pump, an auto-sampler, and a thermo-stated column compartment was used. Chromatographic separations were carried out on an Agilent ZOR-BAX Eclipse SDB-C18 column (4.6 × 250 mm, 1.8 μm). The mobile phase was composed of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) by the following program: 0-1 min, isocratic gradient 15-15% (A); 1-10 min, linear gradient 15-40% (A); 10-16 min, isocratic gradient 40-40% (A); 16-20 min, linear gradient 40-75% (A). The flow rate was set at 0.4 mL/min.

Cell cultures

Human vein endothelial cells (HUVECs) were cultured on 0.2% gelatin-coated T75 flask maintained in culturing medium (M199) supplemented with 20% fetal bovine serum, 90 mg/mL heparin sodium salt, 20 μg/mL endothelial cell growth serum (ECGS), 100 U/mL penicillin and 100 μg/mL streptomycin, at 37°C in a water-saturated 5% CO₂ incubator.

Nitric oxide colorimetric assay

Cultured HUVECs were seeded onto a 96-well plate (1 × 10⁴ cells/well) in 100 μL of Lonza Endothelial Growth Medium (EGM) Bullet kit for 24 hours. In the next day, the medium was replaced by 0.1 mL serum and growth factor free medium. After 2 hours, drugs were applied onto cultured cells. The concentration of NO in the culture medium was detected with the NO kit 55 (Biovision, CA) according to the manufacturer's instructions. NO was rapidly oxidized to nitrite and nitrate, which were utilized to quantify NO production. The amount of nitrate was determined by converting to nitrite, followed by colorimetric determination of total concentration of nitrite as a colored azo dye product of Griess reaction that absorbed at 540 nm using a microplate reader [4].

Laser confocal fluorescence microscopy

Fluorimetric measurements were performed on cultured HUVECs using an Olympus Fluoview FV1000 laser scanning confocal system (Olympus America Inc., Melville, NY) mounted on an inverted Olympus microscope, equipped with a 10X objective. Intracellular NO production and Ca²⁺ concentration were monitored using fluorescent NO indicator 4-amino-5-methylamino-20, 70-difluorofluorescein (DAF-FM DA; from Invitrogen, Grand Island, NY) and fluorescent Ca²⁺ indicator Fluo-4 AM (Invitrogen), respectively [4]. The amount of NO or Ca²⁺ was evaluated by measuring the fluorescence intensity excited at 495 nm and emitted at 515 or excited at 488 nm and emitted at 525 nm, respectively. Changes in intracellular NO production and Ca²⁺ were displayed as a ratio of fluorescence relative to intensity (Fn/F0). Normal physiological saline solution (NPSS) contained 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and 5 mM HEPES (pH 7.4), while Ca²⁺ free solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 5 mM HEPES, pH7.4 and 0.22 mM EGTA. The detection method was reported, previously [5].

Western blot assay

The phosphorylations of eNOS (S1177) and Akt (S473) were determined by western blot assay. Cultured HUVECs were serum- and growth factor-starved for 3 hours before the drug application. After drug treatment, including all the activators, the cultures were collected immediately in lysis buffer (125 mM Tris-HCl, 2% SDS, 10% glycerol, 200 mM 2-mercaptoethanol, pH 6.8), and the protein were subjected to SDS-PAGE analysis. After transferring the proteins to membranes, the membranes were incubated with anti-phospho-eNOS S1177 (Cell Signaling, Danvers, MA) at 1:1,000 dilution, anti-phospho-Akt S473 (Cell Signaling) at 1:5,000 dilutions at 4°C for overnight. Following incubation in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies in 1:5,000 dilutions for 3 hours at room temperature, the immune-complexes were visualized by the enhanced chemiluminescence (ECL) method (Amersham Biosciences, Piscataway, NJ). The band intensities in control and agonist-stimulated samples, run on the same gel and under strictly standardized ECL conditions, were compared on an image analyzer, using in each case a calibration plot constructed from a parallel gel with serial dilutions of one of the samples.

Statistical analysis and other assays

Protein concentrations were measured routinely by Bradford's method (Hercules, CA). Statistical tests were done by using one-way analysis of variance. Data were expressed as mean ± SEM, where n = 3 - 4. Statistically significant changes were classified as significant (*) where *p* < 0.05, more significant (**) where *p* < 0.01 and highly significant (***) where *p* < 0.001.

Results

The quality control of DBT or DBT_{Δcal} was performed on HPLC, and the validation of the calycosin fraction was confirmed on LC-MS/MS, as reported previously [14]. All DBT decoctions were qualified, and the amounts of chemical markers were determined before [14]. One gram of DBT dried powder contained 809.44 μg ferulic acid, 693.19 μg calycosin, 164.58 μg formononetin and 212.01 μg of Z-ligustilide, which had been described previously [14]. From our previous data, calycosin was the only varying parameter in

DBT_{Δcal} as compared with authentic DBT [14]. The amount of calycosin within DBT_{Δcal} was 19.40 μg/g, i.e. over 97% depletion from authentic DBT. In addition, a LC-MS fingerprint was developed for DBT quality control (Supplementary Figure 1).

To quantify the amount of NO production, we utilized Griess reagent. Here, vascular endothelial growth factor (VEGF) served as a positive control, which was capable of inducing NO production in a dose-dependent manner in cultured HUVEC: the maximal induction was at ~2 folds of increase at 20 ng/mL (Supplementary Figure 2). Application of DBT induced NO production in a dose-dependent manner: the maximum induction was at ~200% of increase at ~0.5 mg/mL DBT (Figure 1). The calycosin-depleted DBT (DBT_{Δcal}) however showed a robust reduction in inducing NO production. Extra-addition of calycosin in DBT, i.e. DBT_{+cal}, caused an up regulation of NO production to ~450% of increase (Figure 1). Interestingly, calycosin alone did not affect significantly NO production.

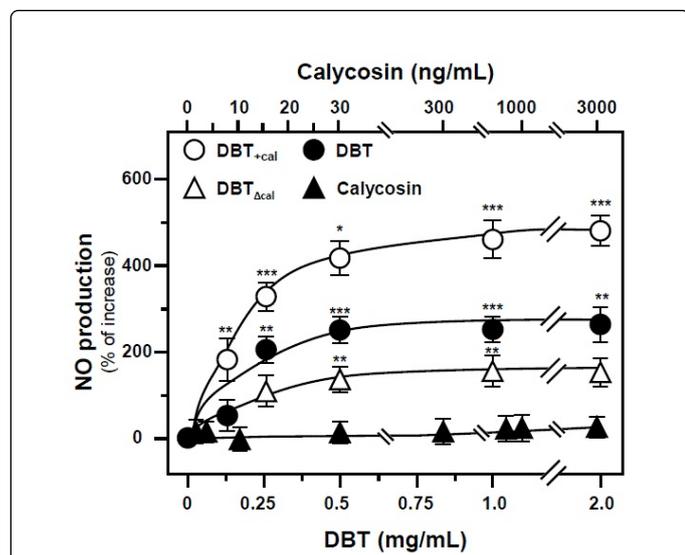


Figure 1: DBT induces NO production detected by Griess reagent. HUVEC cells were pre-treated with serum and growth factor free medium for 3 hours, and then HUVECs were treated with calycosin, DBT_{+cal}, DBT and DBT_{Δcal} at different concentrations for 10 min. Total NO production was calculated from a calibration curve generated from standards provided by the manufacturer and normalized by the protein content of corresponding sample. Data are expressed percentage of increase compared with control, Means ± SEM (n = 4). * *p* < 0.05, ** *p* < 0.01 and *** *p* < 0.001, compared to control.

Laser confocal was another method to detect NO production. This non-fluorescent dye was de-acetylated to become weak fluorescent, which formed strong fluorescent signal after reacting with NO [5]. After drug treatment, the real time fluorescent image was monitored in HUVECs. DBT was able to induce NO production in a time-dependent manner (Figure 2). The maximal stimulation was ~600% of increase in the treatment of DBT_{+cal}, which was much stronger than that of DBT and DBT_{Δcal} (Figure 2). Similarly, the depletion of calycosin reduced the DBT-induced NO signal by half. Similar to the assay by Griess reagent, calycosin alone did not induce NO signal. VEGF, served as a control, induced the signal in a time-dependent manner (Figure 2).

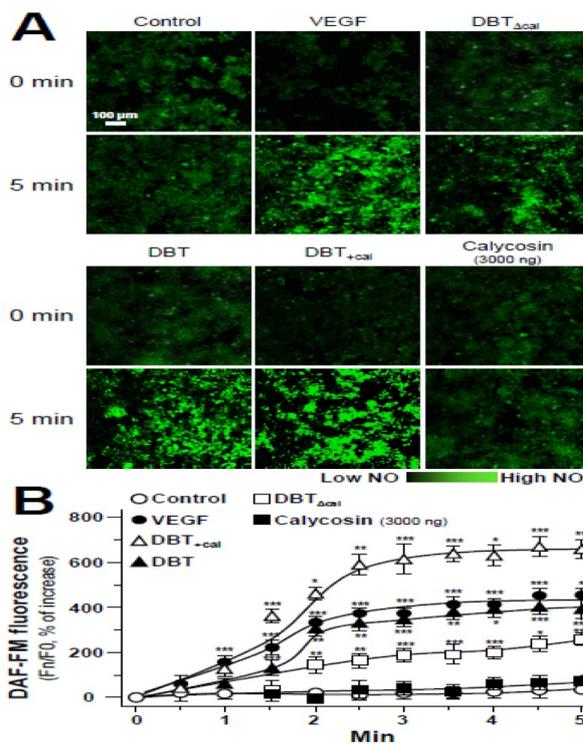
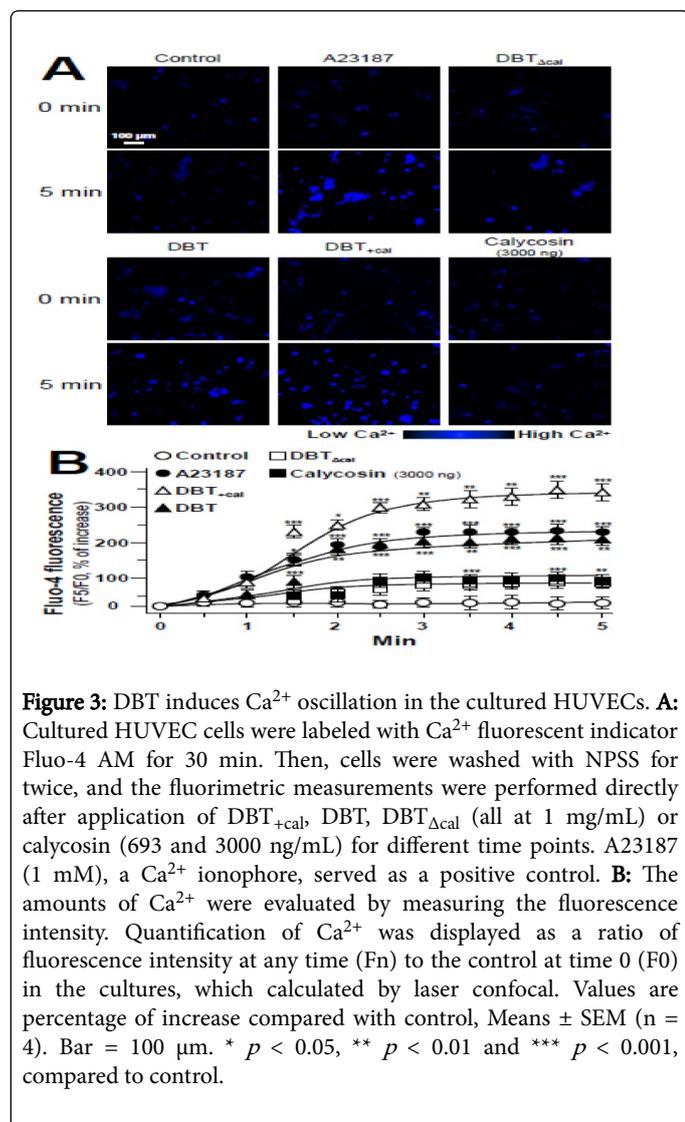


Figure 2: DBT induces NO production detected by DAF-FM DA. **A:** Cultured HUVEC cells were pre-treated with serum and growth factor free medium for 3 hours before labeling with NO fluorescent indicator DAF-FA DA for 30 min. After labeling, cells were washed with NPSS for twice, and the fluorimetric measurements were performed after application of DBT_{+cal}, DBT, DBT_{Δcal} (all at 1 mg/mL) or calycosin (693 ng/mL, 3000 ng/mL) for different time points. VEGF (20 ng/mL) used as a positive control. **B:** The amounts of NO were measured by the fluorescence intensity. Quantification of NO production was displayed as a ratio of fluorescence intensity at any time (Fn) to the control at time 0 (F0) in the cultures, which calculated by laser confocal. Values are percentage of increase compared with control, Means ± SEM (n = 4). Bar = 100 μm. * *p* < 0.05, ** *p* < 0.01 and *** *p* < 0.001, compared to control.

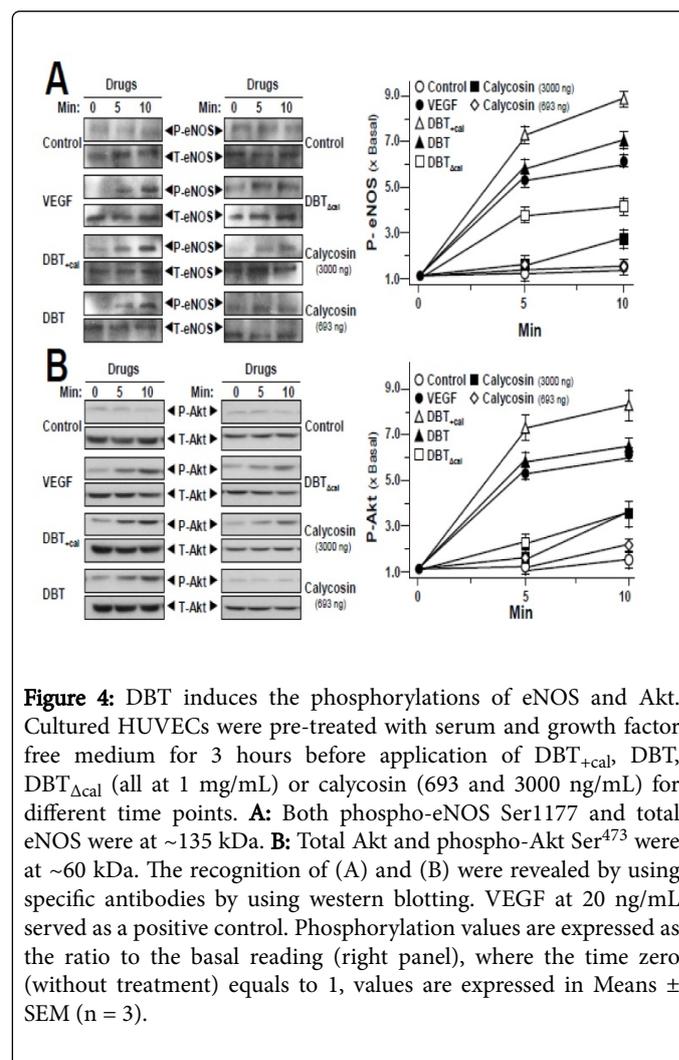
The activation of constitutive nitric oxide synthase (NOS) was depending on the presence of various cofactors and on a conformational change, induced by interaction of calcium-calmodulin (Ca²⁺/CaM) complex [18]. Previous study demonstrated that intracellular free Ca²⁺ induced the activation of eNOS, indicating that the NO production was controlled by cytosolic Ca²⁺ concentration [19]. The intracellular Ca²⁺ mobilization was revealed after application of DBT on cultured HUVECs. Fluo-4 AM, the most common Ca²⁺ indicator, was employed here to monitor Ca²⁺ influx in the cultured cells [4]. A23187 (1 mM), a Ca²⁺ ionophore serving as a positive control, induced Ca²⁺ mobilization in cultured HUVECs in a time-dependent manner (Figure 3). DBT application induced Ca²⁺ mobilization in a time-dependent manner at a maximal of 200% increase (Figure 3). Calycosin-depleted DBT (DBT_{Δcal}) showed a reduction of Ca²⁺ activation by half, as compared to authentic DBT. In parallel to NO production, the addition of calycosin in DBT markedly

increased its activation of Ca²⁺ mobilization; however, calycosin alone showed no effect at all (Figure 3).

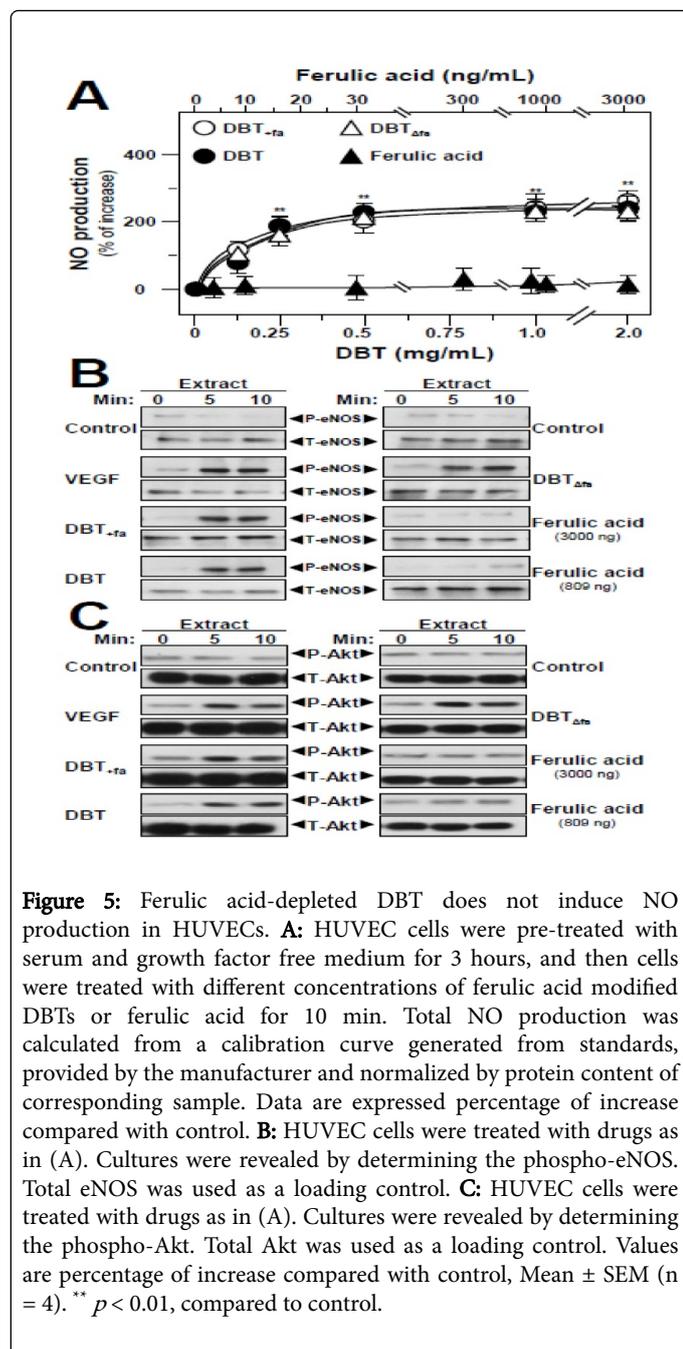


The endothelial isoform of NOS was a key source of production of NO found in cardiovascular system. The production of NO was catalyzed by eNOS. The phosphorylation of eNOS could be at various serine/threonine residues [20]. Of these potential phosphorylation sites, S1177 was a crucial regulator of its enzymatic activity. From western blot results, we found that DBT decoction could induce the phosphorylation of eNOS (Figure 4A). The maximal activation was at ~9 folds at the application of DBT_{+cal}. Comparing to DBT_{+cal} and authentic DBT, DBT_{Δcal} showed the weakest stimulation of eNOS (Figure 4A). VEGF served as a positive control. Calycosin at high concentration could slightly induce the phosphorylation of eNOS (Figure 4A). The activated PI3K/Akt pathway led to phosphorylation of eNOS and increased the production of NO [21]. PI3K/Akt signal transduction pathway is a crucial element in the processes leading to endothelial cell survival induced by VEGF [22]. HUVECs were seeded in 24-well plate with a density of 2×10⁵ cells/mL. Serum and growth factor starvation in cultures were applied 3 hours before any drug or chemical treatment. The phosphorylation of Akt was tested by specific antibodies, i.e. phosphorylation of Akt Ser⁴⁷³ and total Akt. The

obvious increase of Akt activation was observed after the treatment of DBT in a time-dependent manner (Figure 4B). Similar to scenario in eNOS phosphorylation, the depletion of calycosin reduced the DBT-induced Akt phosphorylation; while the addition of calycosin enhanced the phosphorylation to ~8 folds (Figure 4B). VEGF, a positive control, increased the phosphorylation of Akt by ~5 folds. Calycosin at high concentration could only slightly induce the phosphorylation (Figure 4B).



The knock-out approach was further demonstrated to be specific only to calycosin within DBT. Ferulic acid, an active chemical deriving from ASR, was depleted from DBT. The depletion method was same as the scenario in calycosin but just targeting to ferulic acid, and the depletion was over 98% (Supplementary Figure 1). In contrast to DBT_{Δcal}, the ferulic acid-depleted DBT (DBT_{Δfa}) did not alter the DBT-induced NO production in HUVECs, and the extra-addition of ferulic acid (i.e. DBT_{+fa}) showed no addition effect (Figure 5A). In parallel, the DBT-induced phosphorylation of eNOS and Akt did not alter in DBT_{Δfa} and DBT_{+fa} (Figure 5B), suggesting the no role of ferulic acid in endothelial cells.



Discussion

According to history, Egyptian medicine, Babylon medicine, Greek medicine and TCM are always honored as “four major ancient medicine.” Nevertheless, only TCM survived along thousands of years. TCM was summarizing the experience of Chinese to fight against disease and absorbed ideas from classical Chinese philosophy, including Daoism, Buddhism and Confucianism [1]. Based on the above mentioned principles, TCM employed herbal materials, acupuncture and medical message to prevent and mitigate illness. TCM was widely utilized today because of its significantly clinical curative functions. Moreover, researchers have shown great interest in TCM because of its wisdom and beauty from unique medicinal system.

Along with the advancement of modern medicine, complicated pathogen factors have been reported to induce diseases [23]. Thus, a complex disease is hard to be completely cured by current western medicine that is consisting of a synthetic compound and usually is acting on one pathological target [24]. On the other way, synthetic drugs could lead to severe side effects during the treatment [24]. TCM herbal formula composes of at least two or more herbs targeting on various pathogens and developmental stages of disease [25]. Moreover, the combination could be modified according to different stages of illness, even the concrete character of patient. Because of chemical complexity, the action mechanism of TCM formula was not known [3,23,26]. The specific chemical knock-out method, as developed here, could be a novel method to reveal the veil of TCM. By using this strategy, calycosin was illustrated to play a synergistic role in DBT function on endothelial cells; however, calycosin alone had no such function. This result was fully supportive to the rational of making a herbal decoction by mixture.

Besides the cardiovascular properties as described here, we compared the biological efficacies of DBT and DBT_{Δcal} in terms of estrogenic, osteogenic and hematopoietic functions: these activities had a close relationship with menopause [14]. The results indicated that the authentic DBT showed the most robust activation, as comparing to DBT_{Δcal} [14]. Our results suggested that calycosin was one of bioactive chemicals in DBT, which could orchestrate the properties of DBT. In parallel, we had equipped with the same methodology to generate the ferulic acid-depleted DBT, the depletion is over 98%, named as DBT_{Δfa}. By employing same cell model, we had monitored the contribution of ferulic acid within DBT decoction in terms of NO production, our results revealed that there were no significant difference between authentic DBT and DBT_{Δfa}, which suggested that ferulic acid, might possess other biological functions as compared to calycosin. DBT was shown to enhance immune system both *in vitro* and *in vivo* [11,27]. DBT was able to increase the formation of bone marrow, to promote hematopoiesis and thrombopoiesis in immune deficiency mice [27]. In cultured kidney fibroblasts, DBT was shown to induce the expression of erythropoietin via hypoxia-inducible factor up regulation [17]. Polysaccharide was one of well-known substances to boost immune system [11]. Polysaccharide, derived from DBT, was proposed to induce IL-2, IL-8 and IL-10 release in cultured T-lymphocytes [11]. These results proposed a notion of different chemicals within DBT acting synergistically to trigger its clinical efficiency, which accorded the beauty of TCM application.

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