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Anti-inflammatory Effect of Salvia Miltiorrhiza is Mediated via IL-6, JAK, and STAT Pathway in a Dysfunctional Vascular Endothelial Cell

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Abstract

The vascular complication of diabetes mellitus is a problem for the patient, and the ability to cope with the disease and the associated inflammation is a critical aspect of diabetes. Cytokines-induced inflammation in vascular endothelial cells (VECs) plays an active role in chronic diseases such as atherosclerosis, diabetes mellitus, kidney injury, and stroke. We investigated the role of total salvianolic acids (TSA), total tanshinones (TTSN), and their combination (TSA/TTSN) on the activated vascular endothelial cell and its inhibitory effect on signal transduction and cytokines regulation. In the extracellular medium of the injury model of human umbilical vein endothelial cells (HUVECs) induced by thrombin, the human IL-6, VCAM-1, and ICAM-1 were significantly elevated ($p < 0.05$). However, suppression in the TSA, TTSN, and TSA/TTSN (100 µg/L)-treated groups ($p > 0.05$) were notable. TSA alone but not TTSN and TSA/TTSN combination, inhibited the expression of P-selectin ($p < 0.05$) and E-selectin ($p < 0.01$) respectively, in VECs. Western blot analysis showed JAK and STAT expression in VECs however, the protein expression was modest in the Salvia miltiorrhiza-treated groups, indicating the potential of TSA/TTSN in the inflammatory pathways of IL-6, JAK, and STAT signal transduction in endothelial cells (ECs). This study has made novel observations regarding the components of Salvia miltiorrhiza regulatory effect on cytokines in Vascular Biology.

Keywords*: Atherosclerosis; Cytokines; Diabetes mellitus; HUVECs; Inflammation; Salvia militiorrhiza.*

Introduction

The endothelial is the thinnest layer of the cell covering the conduit of the blood vessel, providing a surface by which blood circulates in the lumen. The endothelial cells play a role in the regulation of inflammation, platelet aggregation, and vascular smooth muscle proliferation [1, 2]. Endothelial cells also play special roles, including blood clotting, angiogenesis, blood pressure regulation, the selective barrier between the vessel and the surrounding bloodstream [3].

However, endothelial cells are first targets during dysregulation of cytokines production under chronic disease conditions owing to their important roles, including continuous contact with the immunological system, result in barrier dysfunction and monolayer permeability by triggering endothelial cell-shape change, and cytokines infiltration due to prolonged increase in permeability of the vascular endothelial barrier mechanism [4]. Vessel damage and inflammation, in most cases, give rise to angiogenesis and tumour development [5].

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Furthermore, inflammation and dysregulation of cytokine production are directly involved in the pathogenesis of atherosclerosis, diabetes mellitus, cardiovascular disease, and autoimmune disease [6].

Chronic cardiovascular diseases, coronary artery disease, atherosclerosis, and diabetes mellitus involve complex inflammatory mediators, including endothelial dysfunction right from the onset of the disease [7, 8] Researchers found that the endothelial cell is a critical pathological target in a chronic diabetes mellitus patient, as diabetes poses detrimental havoc to the vascular endothelial cell during disease progression [9].

Hyperglycemia is a pathological rise in blood sugar that occurs in people with diabetes. When the endothelium is exposed to hyperglycemia, including the high glucose-induced impaired antioxidant defence, an array of negative intracellular events promotes endothelial dysfunction and cardiovascular clinical manifestation in diabetes [10]. The loss of endothelial function could also trigger diabetes microvascular (retinopathy, nephropathy, neuropathy) and macrovascular diseases (ischemic heart disease, stroke, peripheral vascular disease) [11].

An enzyme, thrombin (coagulate factor II), is involved in the coagulation cascade and inflammation and exerts its effect on endothelium when there is an imbalance in the coagulation system, causing endothelial dysfunction and stimulating the release of inflammatory mediators and growth factors [12, 13]. Thrombin promotes platelet activation and aggregation, triggers a pro-inflammatory reaction, and has cell membrane receptors abundant in arterial vessel walls [13]. Consequently, thrombin induces leukocyte adhesion on the endothelial surface and subsequent penetration into the underlying tissue resulting in the disruption of the normal biochemical pathway [14]. The cellular effects of thrombin are known to be mediated by protease-activated receptors (PARs), and PAR-1 is involved in the initial platelet aggregation in response to thrombin, while PAR-4 is assumed to contribute to the stability of platelet aggregation [15].

Medicinal plants have numerous bioactive compounds and display various pharmacological activities such as potency, smoother actions, and tolerance, which are clinically relevant criteria for treating chronic conditions such as cardiovascular diseases; (hypertension, diabetes mellitus, atherosclerosis, myocardial infarction), and the accompanying detrimental inflammation [16]. However, the lack of in-depth knowledge of their therapeutic principles limits their application in the clinic and acceptance in the field of medical science.

Researchers in the field of natural medicine (NM) has made tremendous progress regarding the therapeutic use of the dry root of Salvia militiorrhiza, a popular herbal remedy for treating cardiocerebrovascular disease in China [17, 18]. Salvia militiorrhiza is known for promoting blood flow and resolve stasis [16]. The phytochemical report showed that Salvia militiorrhiza is made up of the abietane type-diterpene quinones pigment (lipid-soluble) Tanshinone I, IIA, IIB, Cryptotanshinone, and Dihydrotanshinone, etc. as well as the phenolic group (water-soluble) Danshensu, Salvianolic acids, lithospermic acid, etc. [19, 20]. Furthermore, diterpene quinones and the phenolic group have been shown to have an antiplatelet effect [21]. Research indicates that Salvia militiorrhiza could dissolve thrombosis and improve blood circulation to resolve inflammation [22], but the underlining mechanism involved in these actions is not entirely understood.

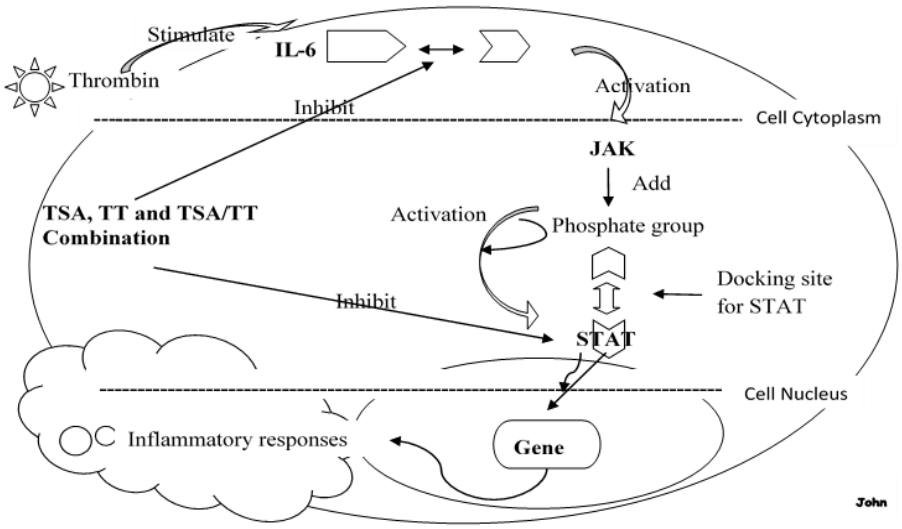

Figure 1. Signal Transduction Pathway of TSA, TT, and TSA/TT Combination Traced from the Activated IL-6, JAK, and STAT

Graphical Abstract

The signal transduction pathway of TSA, TT, and TSA/TT combination traced from the activated IL-6, JAK, and STAT.

TSA, TT, and their combination inhibited thrombin mediated inflammation and blocked the activation of IL-6 outside the cell to stop the binding and activation of the JAK/STAT signal transduction pathway in HUVECs.

The objective of this study is to establish a thrombin injury model of Endothelial Cells (ECs) from Human Umbilical Vein (HUV) in order to study the anti-inflammatory and mechanism of *Salvia militiorrhiza* components (total *Salvianolic acids* and total *Tanshinones*, by therapeutic targeting the pro-inflammatory cytokines.

We would like to state here that this work is original, and the results presented have not been submitted previously in whole or part for publication in another journal nor published in any form whatsoever.

Materials and Methods

Cell Culture

All reagents used for the experiment, including the cell, are bought from ScienCell Research Laboratory, USA. Based on the manufacturer's protocol, the HUVECs (Cat. No. 8000) were incubated with Endothelial Cell Medium (Cat. No.1001) containing 5% FBS (Cat. No. 0025), 1% Penicillin/Streptomycin solution, (Cat. No.0503), 1% Endothelial Cell Growth Supplement (Cat. No. 1052) and pH 7.4. Cells were plated and grown in a 75mm Corning tissue culture flask (Corning Costar Corp., Bedford USA), coated with human plasma fibronectin (Lot. No. NMM1701224, Millipore, USA). Cells were incubated (at 5% CO_2/95% humidified air and temperature of 37°C) and grown to 75 to 80% confluence within 3 to 4 days. At confluence (day 4), cells were trypsinized (Trypsin/EDTA, Cat. No.0103, ScienCell, USA) and the cell pellet were re-suspended in the full endothelial cell medium (ECM) containing 5% FBS and 1% growth factor, then seeded at $6x10^3$ cells in 96-well fibronectin-coated Corning tissue culture plate at an insert volume of 200 µl/well. HUVECs monolayer was used within 24 to 48h at passage 2 to 3 in all the experiments. All procedures were carried out under standard laboratory conditions and safety practices.

Drugs

Two chemical components of Total *Salvianolic acids* [TSA] and Total *Tanshinons*

[TT] were purchased from Xi'an Biotech Inc. P.R. China. A stock solution of *Salvianolic acids* was made using basal ECM (1mg/mL), whereas Tanshinones was dissolving in DMSO (0.1%) and then added to ECM to get the final stock concentration of 1mg/mL). Both were individually filtered through a sterile 0.22µm filter. Thrombin from Bovine plasma (1000U, Cat. No. T8021) was purchased from Solarbio.

Cell Viability Assay

To determine whether TSA, TT or TSA/TT combination is cytotoxic to HUVECs, we performed a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT, Solarbio) assay was performed in order to ensure the therapeutic safety of the drug on the viability of the cell. Briefly, HUVECs were seeded at $1x10^4$ cells/well density and grown to confluence in 96-well culture plates then washed twice with PBS followed by incubation with different concentrations of TSA, TT, and TSA/TT (12.5µg/L, 25µg/L, 50µg/L, and 100µg/L) for 6 hrs without FBS, ECGS, and P/S. The endothelial cells were labelled with 0.5 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) in PBS for 4hrs, and the resultant formazan was dissolved in 0.04N HCL/isopropanol. The absorption was measured using a microplate reader at 570 nm. The endothelial cell viability was expressed as a percentage of the value of the control wells. There are six replicates in each treatment group. The experiment was repeated five times on different occasions.

Cytokine Secretion of the HUVECs Injury Model

HUVECs were grown to confluence in a fibronectin-coated 96-well tissue culture plate. It was washed twice with warm (37°C) PBS. Before thrombin addition, cells were fasted of FBS and ECGS for 15min and treated with 10u/mL thrombin. Based on the various groups, 100 µg/L TSA, 100 µg/L TT, and 100 µg/L TSA/TT combination was added and incubated for 6h at 37°C, 5% CO_2/95% humidify-air. Cells incubated with basal medium alone were taken as control. After 6h incubation with the various treatments, HUVECs culture supernatant was harvested and stored at -70°C. According to the Manufacturer's Instruction, human IL-6, VCAM-1, ICAM-1, P-Selectin, and E-Selectin levels in culture supernatants were measured using ELISA kits (R & R&D System), according to the manufacturer's instruction. Briefly, ten standard wells were set up separately on the ELISA micro-titter plate's wells coated with the corresponding antibodies to make a solid face antibody. Standards were constituted according to the manufacturer's instructions; two wells were set apart as blank. 40µL of sample diluent was pipetted into each well, containing 10µl of the sample, and carefully mixed to avoid bubbles, bringing the final dilution to 5-fold. The plate was covered and then incubated for 30 min at 37°C. Thereafter, 300µl of wash solution (30-fold dilution) was pipetted into each well after the content was discarded, then left for 30 s before draining. This step was repeated five times. After that, 50µL of HRP-Conjugate reagent was added to each well and incubated at 37°C for 30min, after covering with the lid. The washing steps were repeated, followed by the addition of a colorimetric solution (Chromogen A and B). We incubated the plate (37°C for 15min) in the dark. Finally, we added a stop solution (50 µL/well) to each well to stop further reactions. We then measured the absorbent at 450 nm, taken the blank wells as zero.

Western Blotting Analysis

Western blot was performed to quantify the expression of JAK-2 and STAT-3 protein in HUVECs. Briefly, a confluence monolayer of HUVECs was assigned to experimental treatments (Control, Model, Inhibitor, and *Salvia militiorrhiza* group). Cells were incubated for 6h at 37°C, 5%CO_2 / 95% moisturized air with basal medium, basal medium /10units/mL thrombin, 25µM AG490

(AG490, Lot. No. 120M1985, Sigma-Aldrich), and 100µg/L TSA/TT + 10u/mL thrombin respectively for the Control, Model, Inhibitor, and Drugs groups. Protein was extracted from the cell for western blot 6hrs after incubation. According to the Manufacturer's Instruction, cells were washed and lysed using a cell lysis kit (Sangon Biological Engineering Technology & Service Co., Ltd., Shanghai, China). Lysates were sheered repeatedly and then centrifuged at 12,000xg for 5min at 4°C. BCA protein concentration assay was done according to the manufacturer's instruction (BCA protein assay kit, Product No. SK3021-500, Sangon Biological Engineering Technology & Service Co. Ltd., Shanghai, China), in order to verify the volume of protein to be loaded into the Gel. An equal volume of sample buffer (42% 0.5 mol/L Tris [pH 6.8], 42% glycerol, 55 bromophenol blue, and 1%SDS; Bio-Rad Laboratories, Inc. Hercules, CA, USA) was added to the sample. Test samples were loaded onto 5-8% (v/v) Sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) and run until the blue marker reached the end of the gel. Proteins are transferred to Immobilon®-P (PVDF membranes, Cat. No. IPVH00010, Millipore Corp., Billerica, USA). All membranes were blocked for non-specific binding by washing in Tris-buffer saline (25mM Tris, pH 6.8, TBST) containing 5% w/v skim milk and 0.1% v/v Tween-20 for 2h at room temperature. The membrane was incubated overnight at 4°C with primary antibodies, Anti-STAT3 (Cat. No. 44384G, Invitrogen), and Anti-JAK2 (Cat. No. 06-255, Millipore) at a dilution of 1:100 to 1: 1500 according to the manufacture's instruction. After three washed, the blot was incubated for 1h at room temperature with horseradish peroxidase-conjugated goat-anti-rabbit IgG (Beijing Biosynthesis Biotechnology Co., Ltd. China) at dilution of 1:1000 in TBST. Thereafter, the washing step was repeated, and the antigen-antibody complexes were detected using enhanced chemiluminescence (ECL, Lot. No. 4622841, GE Healthcare Bio-Sciences AB Sweden) reagent and visualized by scanning with the Bio-Rad Versa Doc (Bio-Rad, Richmond, CA USA) equipped with Nikon Imaging camera. We quantified the band density using Quantity One version 4.2.2 software (Bio-Rad, USA). β-actin (Rabbit polyclonal anti-β-actin, Santa Crus Biotechnology CA, USA) was used as a protein standard.

Statistical Analysis

We expressed all data as the mean ± SD of results obtained from at least three individual experiments performed in duplicate using SPSS version 11. A p-value < 0.05 was regarded statistically significant, while a p-value < 0.01 was considered highly significant.

Results and Discussion

Effect of TSA, TT, and TSA / TT on Cell Viability

Effect of TSA, TT, and TSA / TT on cell viability of HUVECs was determined using the modified MTT assay, which showed no cytotoxicity, indicating that the incubation at the range of 12.5 µg/L to 100 µg/L for 6hrs is suitable for the viability of the cell compared with the model, Figure 2 and 3.

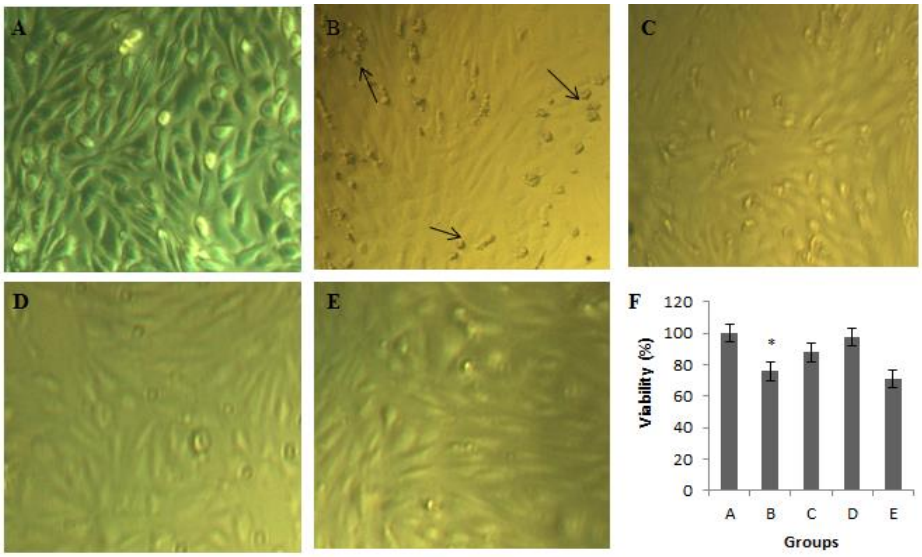

Figure 2. Micrographs of HUVECs taken using a Phase-Contrast Microscope Equipped with a Digital Camera (X100)

Micrographs of HUVECs taken using a phase-contrast microscope equipped with a digital camera (X100). A: Control, B: Model (incubated with basal medium only), C: incubated with thrombin + TSA, D: incubated with thrombin + TT, E: incubated with thrombin + TSA/TT, and F-panel represent viability chart of HUVECs, incubated with thrombin and Salvia militiorrhiza components for 6 hrs. Black arrows in panel B indicate cell injury. Notice the tumor-like swellings and distortion from the normal morphology of HUVECs 6 hrs after incubating with thrombin (Figure 2).

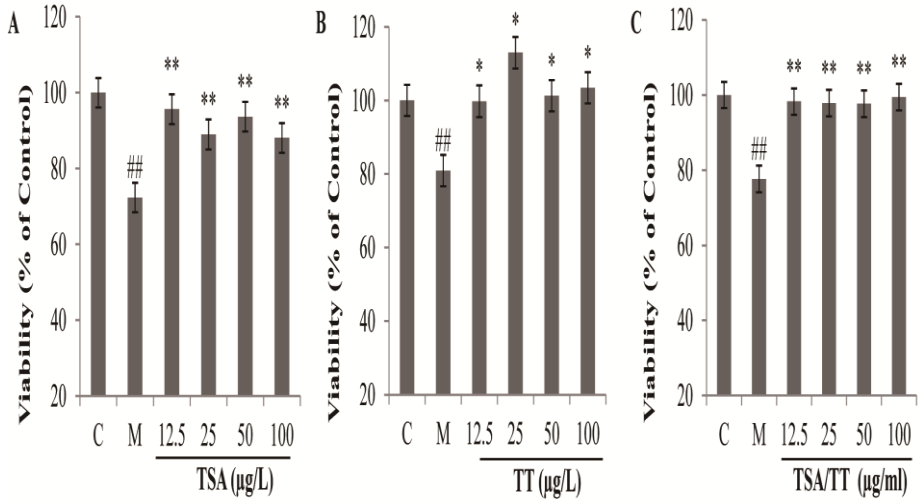

Figure. 3. The Viability of HUVECs after Incubation with TSA, TT, and TSA/TT for 6 hrs

The Viability of HUVECs after incubation with TSA, TT, and TSA/TT for 6 hrs. A: total salvianolic acids (TSA); B: total Tanshinone (TT); and C: total salvianolic acid/total tanshinone (TSA/TT). Lower panel: C represents Control, and M represents Model. A, B, and C are low to moderate doses of TSA, TT, and TSA/TT, respectively. Notice the trend in the low to moderate doses. TSA, TT, and TSA/TT at low to moderate doses enhanced the cell viability (Figure. 3).

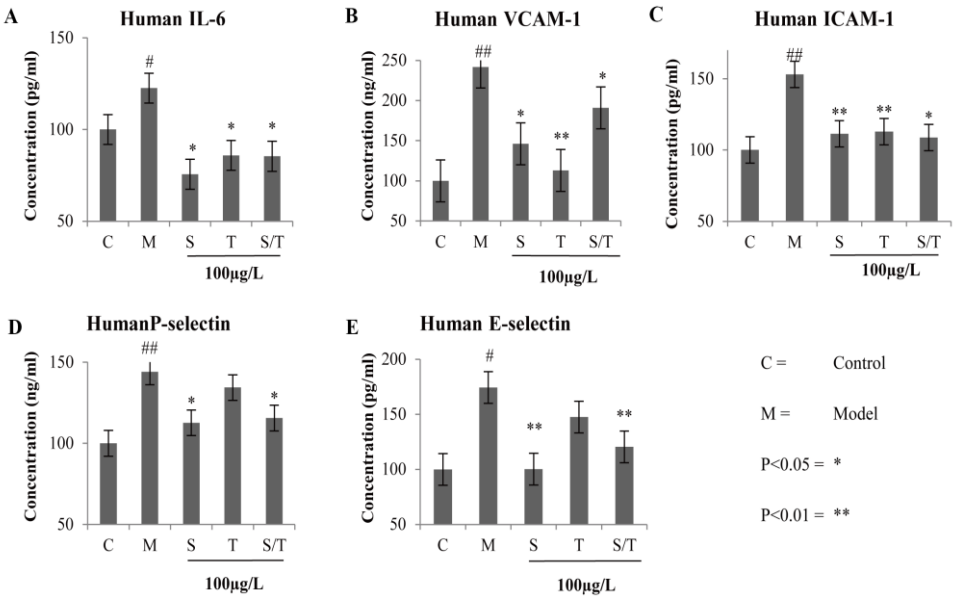

Figure 4. Effects of Salvia Militiorrhiza Components on Cytokines Secretion

Effect of TSA, TT, and TSA/TT Combination on Cytokine Secretion of HUVECs

Compared with the Control, marked significant differences were observed in cytokines secretion of HUVECs incubated with thrombin within 6 hrs of incubation; IL-6 (P<0.05), VCAM-1, and ICAM-1 (P < 0.01); P-selectin (P < 0.01), and E-selectin (P < 0.05) respectively. It is interesting to note that the inflammatory mechanism as related to atherosclerosis and diabetes mellitus is the activation of endothelial cell [23, 24]. Platelet activation cascade leads to cellular activation of P-selectin, and increase ICAM-1, VCAM-1, and E-selectin; and result in inflammatory progression, endothelial dysfunction, and the formation of the atherosclerotic lesion (Figure 4) [25-27].

S: Salvianolic acids, T: Tanshinones, S/T: the combination of Salvianolic acids and Tanshinones. S, T, and S/T significantly (p < 0.05 and p < 0.01) ameliorated human IL-6, human VCAM-1 and human ICAM-1, respectively, whereas S (p < 0.05) and S/T (p < 0.01) also significantly ameliorated human P-selectin and human E-selectin, respectively, contrary to the Tanshinones alone.

Interestingly, *Salvia militiorrhiza* components significantly ameliorated the expressions of IL-6 (p < 0.05), VCAM-1 (p < 0.05), (p < 0.01), (p < 0.05) and ICAM-1 (p < 0.01), (p < 0.01), (p < 0.05), across the groups for TSA, TT, and TSA/TT respectively, Figure 3. Furthermore, the expression of cell adhesion molecules, P- and E-selectin, in thrombin-induced HUVECs injury is an indication of leukocytes rolling on inflamed endothelium [28]. This was mitigated by the addition of *Salvia militiorrhiza* components. TSA (P-selecting, p > 0.05), TSA/TT (P-selectin, p > 0.05) and TSA (E-selectin, p > 0.01), TSA/TT (E-selecting, p > 0.01) levels respectively in thrombin-induced injury of HUVECs were greatly mitigate by *Salvia militiorrhiza* components at 100μg/L (raw dose), Table 1. TT alone have a modest effect on P- and E-selectin (p > 0.05) and (p > 0.05), respectively. Therefore, in view of this, there is a high probability that TSA alone or TSA/TT combinations are potent components of *Salvia militiorrhiza* in regard to cytokines regulation of endothelial cells if tested clinically as preventive or curative in patients vulnerable to diabetes mellitus or atherosclerosis.

Western Blotting

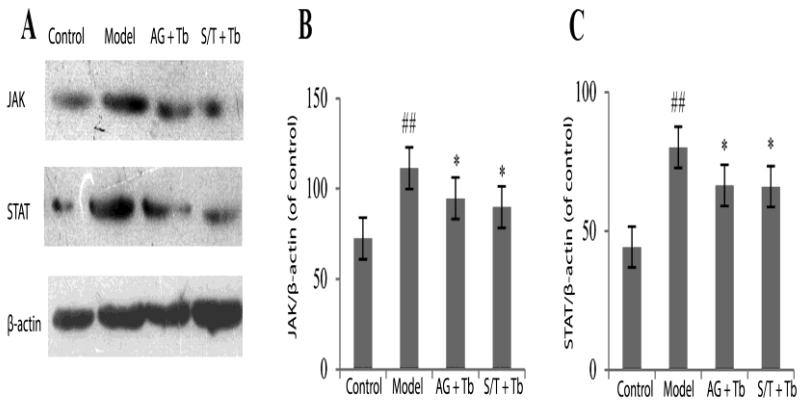

Figure 5. Effect of Salvia Militiorrhiza Components on the Expression of JAK and STAT-3 Proteins

A: Original blot images for JAK, STAT, and β-actin; B and C: Quantification of the blot image of panel A. Control (healthy HUVECs), Model: (HUVECs thrombin Injury Model), AG+Tb: (HUVECs incubated with AG490+Thrombin), S/T+Tb: (HUVECs incubated with Total Salvianolic acids + Total Tanshinons + Thrombin). n = 3, $p < 0.05$.

Thrombin initiated signal transduction in HUVECs as shown in Figure 5, model. AG 490 is a potent inhibitor of epidermal growth factor tyrosine kinase activity, JAK family tyrosine kinases, and guanylyl cyclase. Epidermal growth factor tyrosine kinase found in human platelets, macrophages, plasma, etc. act by binding to JAK on the cell surface to stimulate the tyrosine kinase activity protein of the receptor, which initiates a signal transduction cascade after binding, and results in a variety of biochemical changes within the cell. The addition of AG 490 to the cells significantly diminished JAK and STAT. This is comparable to TSA/TT as shown by the blot analysis, indicating that TSA/TT attenuated the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway in HUVECs to ameliorate the dysfunctional production of inflammatory cytokines [29, 30]. Thus represents one of the novel mechanisms by which TSA/TT acts to protect the cells against inflammatory progression in atherosclerotic and diabetes mellitus event, making *Salvia militiorrhiza* a versatile herbal drug in atherosclerosis and diabetes [31].

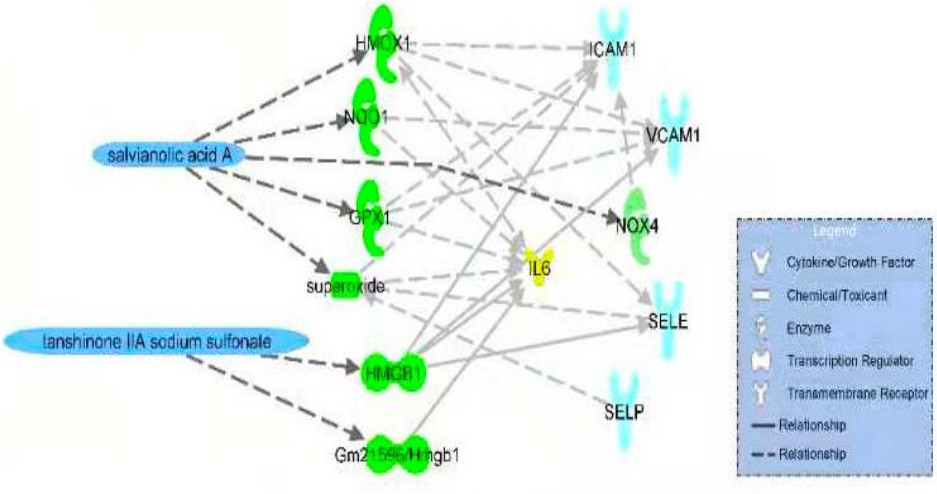

Figure 6. Ingenuity Pathway Analysis (IPA)

An updated view of biological pathways and functions connecting the active components of *Salvia militiorrhiza* and dysfunctional inflammatory signal transduction using ingenuity pathway analysis (IPA). Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA) was performed to trace connections of the cytokines with inflammation and to determine how the different cytokines expressed in HUVECs were regulated by *Salvia militiorrhiza* components. Salvianolic acid A have connections with IL-6; ICAM1, VCAM1 (leucocyte rolling and adhesion), SELE, and SELP (cell adhesion molecules and platelet activation) through Enzymes (HMOX1, NOO1, GPX1), and Superoxide (chemical/toxicant). Whereas Tanshinone IIA have connections with IL-6, ICAM1, VCAM1 (leucocyte rolling and adhesion), and SELE through transcription regulator (HMGB1 and Gm21596/Hmgb1) (Figure 6).

Conclusion

Dry root of *Salvia militiorrhiza* is one of the most important and widely used herbal medicine for hundreds of years in treating several ailments such as coronary artery disease, myocardial ischemia, and other cardiovascular diseases [16] in China and other countries, but the knowledge of the pharmacological targeting pro-inflammatory cytokines is poorly understood. We hypothesized that at certain doses, TSA, TT, and TSA/TT combination inhibits dysfunctional cytokines secretion to regulate inflammation in endothelial cells. The data demonstrate that TSA, TT, and TSA/TT, 2:1 combination (100 µg/L) inhibited thrombin in culture and blocked the increased expression of IL-6, JAK, STAT signal transduction pathway similar to tyrphostin AG 490 (AG490), and revised the high levels of IL-6, ICAM-1, VCAM-1, P-selectin, and E-selectin respectively, to protected HUVECs against thrombin-induced inflammatory injury and endothelial dysfunction. In this study, *Salvia militiorrhiza* components showed potential in regulating dysfunctional pro-inflammatory cytokines in vascular endothelial cells [16].

Inflammation is a sign of physical or systemic injury involving the signaling of inflammatory mediators, and chronic inflammatory as found in diabetes mellitus, could be harmful to the body [32]. Inflammation is one of the serious and major causes of endothelial dysfunction resulting in atherosclerotic vascular injury and remodeling in diabetes mellitus [32]. The activation of the JAK/STAT signal pathway in vascular endothelial cells results in inflammatory progression and endothelial dysfunction [34]. It was experimentally proven that soluble forms of IL-6, ICAM-1, VCAM-1, P-selectin, and E-selectin are found in the activated endothelial cells [35]; and predict the development of macrovascular disease in a diabetic patient [36]. It is scientifically reasonable to note that an herbal medicine targeted at regulating the pathogenic circulation of inflammatory cytokines is capable of ameliorating the detrimental effects of diabetes mellitus in the patient.

It was experimentally reported that the several components in combination in *Salvia militiorrhiza* ameliorate insulin resistance through the regulation of glucose and fat metabolism [37]. Furthermore, tanshinone IIA, which is a major component in *Salvia militiorrhiza*, is capable of attenuating renal damage in a diabetic model through the inhibition of oxidative stress and inflammatory pathway [38]. This is proof that the dry root of *Salvia militiorrhiza* possesses both anti-inflammatory and antioxidant properties as a relevant therapy in diabetes. From the experimental point of view, these claims are worth analyzing as related effects were observed in the current work. This study has made an observation regarding one of the pathways by which *Salvia militiorrhiza* regulates pro-inflammatory cytokines production in the pathophysiology of arterial disease.

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Declaration

We declare that this work is original and has not been submitted for publication in any journal nor published in any form whatsoever.

Conflict of Interest

The authors of this manuscript declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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