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Anti-inflammatory effects of neferine on LPS-induced human endothelium *via* MAPK, and NF-κβ pathways

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This study aimed to evaluate the anti-inflammatory mechanism of neferine, a bisbenzylisoquinoline alkaloid, on the lipopolysaccharide (LPS)-induced inflammation in Human Umbilical Endothelial Cells (HUVECs) and pulmonary aorta cells. Production of pro-inflammatory cytokines and nitric oxide (NO) was determined using Griess reaction in human endothelial cells. The levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and mitogen-activated protein kinases (MAPKs) were analyzed using real time PCR and western blotting. Neferine significantly prevented the NO, TNF-α, COX-2, iNOS, IL-1B, and other inflammatory mediators formation in increasing dose as compared to LPS-induced human endothelial cells. The expressions of NADPH oxidase subunits $p22^{phox}$, $p47^{phox}$, and $gp91^{phox}$ were increased in LPS-induced HUVECs but neferein was able to reverse the effect in a dose-dependent manner. The anti-inflammatory effects of neferine in LPS-induced endothelial cells are attributed through the modulation of MAPK and NF-κβ pathways. Collectively, these results suggest that neferine could be beneficial in the early treatment of atherosclerosis to prevent stroke and heart disease.

1. Introduction

Atherosclerosis is a condition caused by inflammation in vascular endothelium. The endothelium forms as a separator of vessel lumen and its surrounding tissue for immune responses during inflammatory reaction (Lee et al. 2013). Exposure of endothelium to pro-inflammatory mediators such as lipopolysaccharides (LPS) initiates an inflammatory response through activation of the endothelium which is a leading cause of atherosclerosis (Li et al. 2016). Excessive formation of reactive oxygen species (ROS) due to oxidative stress is also implicated in the pathogenesis of atherosclerosis (Ahn et al. 2010). Two important mediators of vascular inflammation are nuclear factor kappa-β (NF-κβ) and tumor necrosis factor-α (TNF-α). Attenuation of NF-κβ pathway is reported to be an effective measure for prevention of vascular inflammation (Ming et al. 2015). Vascular inflammation is the beginning stage of atherosclerosis progression, hence preventing it could be beneficial to attenuate severe ailments such as stroke and heart disease. Previous studies on naturally derived bioactive compounds with antioxidant activities have shown interesting outcomes on preventive measures of vascular endothelial inflammation (Bae and Bae 2011; Lee et al. 2013; Li et al. 2016). Therefore this study aimed to evaluate the anti-inflammatory mechanism of neferine, a bisbenzylisoquinoline alkaloid, on the lipopolysaccharide (LPS)-induced inflammation in Human Umbilical Vein Endothelial Cells (HUVECs) and pulmonary aorta cells.

Plant derived compounds have been well documented for their therapeutic potentials in modern medicine. Previous studies on bioactive compounds such as flavonoids, alkaloids, and triterpenoids have been reported their anti-inflammatory potential against various disorders including endothelial inflammation (Chao et al. 2013). It is known from earlier studies that bioactive compounds with anti-cancer properties are capable of exerting anti-inflammatory effects on various conditions (Poornima et al. 2013, Park et al. 2015). Neferine is a major bisbenzylisoquinoline alkaloid compound from the seed embryo of Lotus plant (*N. nucifera* Gaertn). Ethnobotanical claims on various parts of the Lotus plant in Chinese medicine include anti-inflammatory properties and as well reduced anxiety (Zhang et al. 2012). The pharmacological relevance of neferine has been evaluated by researchers in various studies. Neferine is reported to possess anti-cancer properties through apoptotic mitochondrial pathway regulation in HSC-T6 cells, enhanced cytotoxicity in anticancer drug treated MCF-7 cells, and induced apoptosis in liver cancer cells (Ding et al. 2011). Pharmacological values of neferine include treatment of hyposomnia, high fever, arrhythmia, obesity, platelet aggregation and thrombosis, and importantly prevention of pulmonary fibrosis (Zhang et al. 2012). Studies also found that neferine has the ability to inhibit the proliferation of hypertrophic scar fibroblasts, vascular smooth muscle cells, and osteosarcoma cells (Poornima et al. 2014). So far, there are no studies on the anti-inflammatory and protective mechanism of neferine on LPS-induced human vascular endothelial cells. Hence, we for the first time report the essential anti-inflammatory mechanism of neferine on LPS-induced induced inflammation in Human Umbilical Endothelial Cells (HUVECs) and pulmonary aorta cells.

2. Investigations and results

2.1. *Effect of neferine on endothelial cell viability*

The cell viability of HUVECs and human pulmonary aorta cells were tested with MTT assay. The results showed that neferine did not exert cytotoxicity to endothelial cells, where LPS induced cells and the control cells were almost similar in cell viability (Fig. 1). There was no significant difference between the groups which proves that neferine did not cause cellular toxicity to HUVEC and pulmonary aorta cells.

2.2. Neferine inhibits LPS-induced NO production

Nitric oxide (NO) productions in endothelial and microglial cells were determined using Griess reaction. It was noticed that LPS-induced cells exerted significantly high ($p < 0.05$) levels of NO as compared to control cells whereas neferine treatment significantly reduced ($p < 0.05$) the levels of NO in endothelial cells (Fig. 1) and microglial cells (Fig. 2). It is notable that neferine is able to reduce the production of nitrite in LPS-induced cells almost to the level of nitrite produced by control cells.

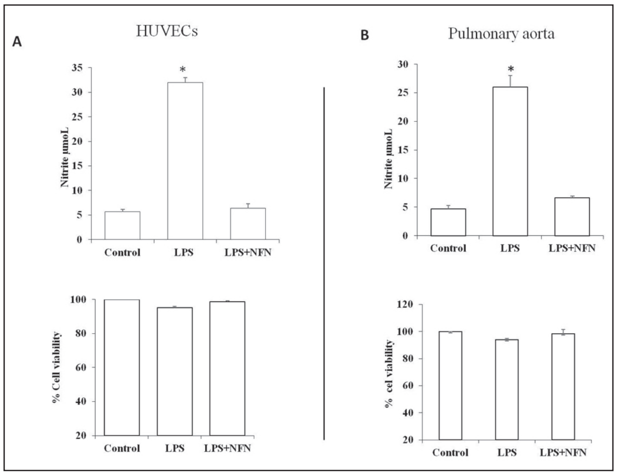

Fig. 1: Effect of neferine on LPS induced NO generation in microglia cells. Cells were pretreated with compounds for 30 min, and then 1 µg/mL of LPS was added and cultured for 24 h. Nitrite generation in culture media (A, HUVECs cell; B, pulmonary aorta) was determined using Griess reaction. The cytotoxicity of compounds was determined by MTT assay (A, HUVECs cell; B, pulmonary aorta). Data represent mean±standard deviation (S.D.) of at least three independent experiments. Symbol mark indicates significantly differences from LPS alone treatment group (*$p < 0.05$).

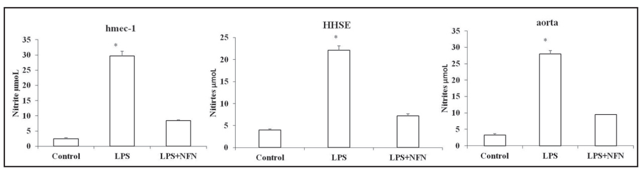

Fig. 2: Effect of neferine on NO generation in activated HAPI rat microglia, macrophage cells and primary astrocytes cells. Microglia cells were pretreated with neferine for 30 min, and then LPS or LPS/IFN-g was added and cultured for 24 h. Nitrite generation in culture media (A, hmec-1, B. HHSE, C. aorta) was determined using Griess reaction. Data represent means±S.D. of at least three independent experiments. Symbol mark indicates significantly differences from LPS alone treatment group (*$p < 0.05$).

2.3. Effects of neferine on tumor necrosis factor-α (TNF-α) production

Pro-inflammatory cytokines have important functions during inflammation which is common in LPS-induced cells. The levels of TNF-α significantly increased ($p < 0.05$) in LPS-induced endothelial cells (Fig. 3) as compared to the control cells. Neferine was able to reverse this condition by dose-dependently suppressing ($p < 0.05$) the production of the pro-inflammatory cytokine TNF-α in LPS-induced endothelial cells. The results indicate that neferine has anti-inflammatory properties against LPS-induced inflammation in cells.

2.4. LPS-induced oxidative stress in endothelial cells

Oxidative stress due to overproduction of ROS is an important mediator of inflammation. ROS production in LPS-induced endothelial cells was analyzed using fluorescent DCFH-DA detection. LPS significantly increased the intracellular ROS production in endothelial cells (Fig. 5A). Neferine was able to inhibit the intracellular ROS production in LPS-induced endothelial cells.

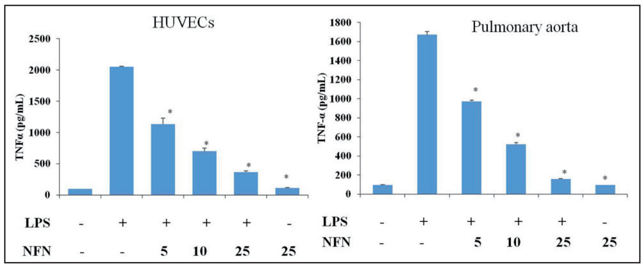

Fig. 3: Effect of neferine on LPS induced TNF-a generation in HUVECs endothelial cells (A) and pulmonary aorta cells (B). Endothelial cells were pretreated with neferine for 30 min, and then LPS was added and cultured for 24 h. TNF-a generation in culture media was determined using ELISA kit. Data represent means±S.D. of at least three independent experiments. Symbol mark indicates significantly differences from LPS alone treatment group (*$p < 0.05$).

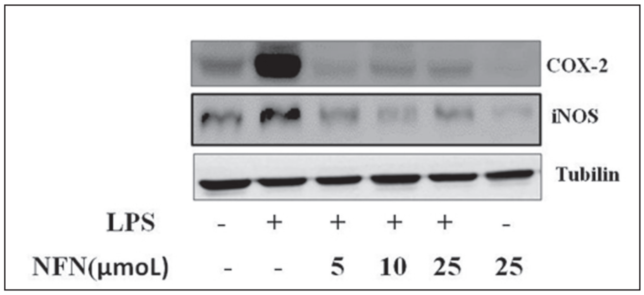

Fig. 4: Effect of neferine on LPS induced pro-inflammatory genes expression in endothelial cells. HUVECs cells were pretreated with neferine for 30 min, and then LPS was added and cultured for 24 h. The mRNA levels of A.TNF-α, B. iNOS, C. COX-2 and D. IL-1b were assessed by qRT-PCR. The protein levels of iNOS and COX-2 were assessed by western blot (E). The α-tubulin was used as an internal control. Data represent means±S.D. of three independent experiments. Symbol mark indicates significantly differences from LPS alone treatment group (*$p < 0.05$).

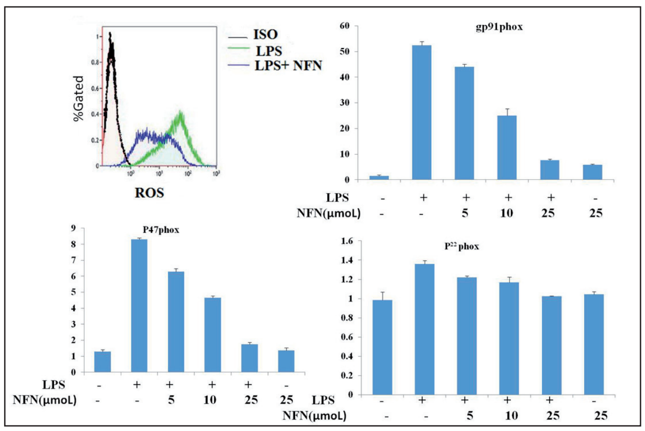

Fig. 5: Impact of neferine on intracellular ROS era and articulation of NADPH oxidase in endothelial cells. HUVECs cells were pretreated with neferine for 30 min, and then LPS was added and cultured for 4 h. After incubation with 10 mM of DCFH-DA, the generation of intracellular ROS was measured by flow cytometry (A). The mRNA expression of gp91^{phox} (B), p47^{phox} (C) and p22^{phox} (D) was determined by qRT-PCR. The relative expression value of control group was set to 1. Data represent means±S.D. of three independent experiments. Symbol mark indicates significantly differences from LPS alone treatment group (*$p < 0.05$).

This result contributes to the supporting fact of anti-inflammatory effects of neferine in LPS-induced vascular endothelial cells.

2.5. Effects of neferine on pro-inflammatory gene and protein expressions

The pro-inflammatory and oxidative stress gene expressions of TNF-α, IL-1β, iNOS, COX-2, gp91^{phox}, p47^{phox}, and p22^{phox} were determined through mRNA levels in endothelial cells using qRT-PCR. Results of mRNA expression in LPS-induced endothelial cells were significantly elevated ($p < 0.05$) as compared to control cells whereas neferine was able to reduce the level of the mRNA expressions in a dose-dependent manner ($p < 0.05$). Pro-inflammatory genes (Fig. 4) as well as NADPH oxidase related sub-unit genes (Fig. 5) were significantly suppressed by neferine in LPS-induced endothelial cells which indicates the ability of neferine to prevent inflammation. Protein expressions of inflammatory mediators COX-2, iNOS, NF-κβ, and MAPK mediators were also determined in LPS-induced endothelial cells through western blotting. The results of western blot analysis on COX-2 and iNOS levels were increased in LPS-induced cells as compared to control cells but the administration of neferine significantly prevented the activities of iNOS and COX-2 in an increasing dose pattern (Fig. 4). The results are in harmony with the level of NO production in LPS-induced cells.

NF-κβ pathway activation is the key in inflammatory process. NF-κβ subunit and transcription factors p65, pIkkα, pIkkβ, and pIkβ-α were determined in LPS-induced endothelial cells with lamin-B and α-tubulin as internal controls. Protein expressions of NF-κβ subunit and transcription factors were increased in LPS-induced endothelial cells compared to control cells (Fig. 6). This shows Ikβ-α was phosphorylated and p65 was translocated into the nucleus to trigger the activation of NF-κβ pathway causing inflammation. Neferine was able to suppress and prevent the activation of NF-κβ in a dose-dependent manner in LPS-induced cells as shown in the results. Anti-inflammatory properties of neferine were further proven through MAPK pathway analysis. The pathways of MAPK which are p38, JNK, and ERK were analyzed in LPS-induced endothelial cells which showed increased protein expressions of phosphorylated MAPK pathways as compared to control cells (Fig. 6). Neferine was able to prevent the phosphorylation of p38, JNK, and ERK dose-dependently, which proves the anti-inflammatory effects of Neferine on LPS-induced endothelial cells.

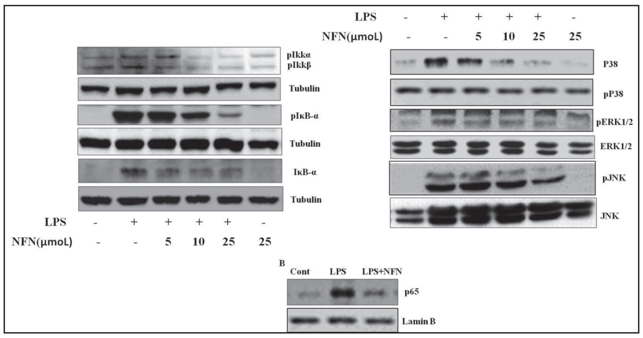

Fig. 6: Impact of neferine on NF-kB initiation in actuated microglia. HUVECs cells were pretreated with neferine for 30 min, then LPS was added and cultured for indicated times. The levels of phospho-Ikka, b (5 min), phospho-IkB-a (10 min) and IkB-a (20 min) were assessed by western blot (A). The α-tubulin was used as an internal control. The expression of p65 (1 h) in nuclear was assessed by western blot (B). The lamin B was used as an internal control. The NF-kB luciferase activity (16 h) was assessed in HUVEC cells stably expressing NF-kB.

3. Discussion

Endothelial cells are important regulators of inflammation whereby directing leukocytes to the site of inflammation upon receiving signals from other mediators and inflammatory gene expressions (Chuang et al. 2014). Endothelial cells are the leading cause of vascular inflammation which contributes to the pathogenesis of atherosclerosis and other serious ailments like heart diseases (Chistiakov et al. 2013). Naturally occurring bioactive compounds are known to possess therapeutic potential against severe inflammatory responses including vascular endothelial inflammation (During and Larondelle 2013; Du et al. 2017). In this study, we found that neferine has the ability to suppress human endothelial cells (HUVECs and pulmonary aorta cells) inflammation induced by LPS. Cell viability test proved that neferine did not cause toxicity to the cells and is considered safe for *in-vivo* tests. The results indicated that the anti-inflammatory potential of neferine was due to its ability to modify the MAPK and NF-κβ pathways in LPS-induced endothelial cells.

The inflammatory response in LPS-induced endothelial cells were similar to common vascular inflammation where the pro-inflammatory cytokine TNF-α was present in high volumes. TNF-α is the vital trigger for inflammatory response and it was found to be excessively present in both LPS-induced endothelial cells. TNF-α has been implicated in the progression of several vascular diseases including atherosclerosis and heart complications (Hsu et al. 2013). Neferine dose-dependently suppressed the TNF-α production as presented in the results which shows the initial anti-inflammatory process in LPS-induced cells. NO is another important enhancer of inflammatory response. LPS-induced endothelial cells triggered the production of NO in excess which is known to be cytotoxic to the cells. This can be related to the cell viability results of LPS-induced cells which were slightly reduced as compared to untreated control cells. Neferine significantly prevented the NO productions in LPS-induced endothelial cells and this could be linked to the suppression of TNF-α production since NO is also an initiator for pro-inflammatory cytokine release through an autocrine system (Amor et al. 2010). Oxidative stress is a well-known condition in the progression of inflammation that leads to numerous diseases. LPS-induced endothelial cells also exhibited an overproduction of ROS in the DCFH-DA fluorescence results. NADPH oxidases are prominent sources of superoxides in vascular endothelial cells. The gp91^{phox} and p22^{phox} are vital membrane bound catalytic components of endothelial NADPH oxidase whereas p47^{phox} is an important cytosolic component for vascular ROS production (Lee et al. 2014). From the results, it was clearly shown that the mRNA expressions of NADPH oxidase subunits were increased in LPS-induced endothelial cells. The increased NADPH oxidase mRNA expressions and ROS production are interlinked in the LPS-induced cells which implicate vascular inflammation. Neferine was able to reverse all these effects in a concentration-dependent manner against LPS-induced endothelial cells. Inhibition of ROS production and reduced mRNA expressions of NADPH oxidase subunits indicates the vascular protective effects of neferine.

Pro-inflammatory mediators IL-1β, iNOS, and COX-2 are commonly present at the site of inflammation (Kim et al. 2012). Similarly, LPS-induced endothelial cells exhibited high mRNA expressions of these inflammatory mediators. Increased concentration of iNOS is related to the levels of NO production and other pro-inflammatory cytokines. Neferine significantly prevented the iNOS, COX-2, and IL-1β levels in a dose-dependent effect in LPS-induced cells. The results were in accordance with the prevention of oxidative stress and anti-inflammatory process of neferine. MAPK and NF-κβ pathways are known to be involved in the regulation of inflammation (Hurks et al. 2014). Once pro-inflammatory cytokines are produced, they immediately trigger the translocation of NF-κβ into the nucleus to initiate transcription process for inflammatory response (Hsu et al. 2013). MAPK pathway could be triggered by oxidative stress which could be detrimental to vascular endothelial cells leading to severe diseases like atherosclerosis. LPS-induced endothelial cells demonstrated highly activated MAPK and NF-κβ pathways through increased concentrations of their relative subunits as shown in results. This could be related to the increased concentrations of pro-inflammatory cytokines and mediators such as TNF-α, IL-1β, iNOS, and COX-2 thus providing evidence for severe vascular inflammation. Prevention of NF-κβ transcription factor and MAPK pathway activation is beneficial in protecting vascular cells from progressing into atherosclerosis and heart diseases. Neferine was able to significantly modify the NF-κβ and MAPK activation pathways in a concentration-dependent manner in LPS-induced endothelial cells. The overall inhibition of pro-inflammatory mediators and suppression of inflammatory mRNA gene expressions by neferine in the LPS-induced endothelial cells can be attributed to the prevention of NF-κβ and MAPK pathways activation.

As a conclusion, neferine has shown potential anti-inflammatory effects against LPS-induced human vascular endothelial cells through the suppression of NF-κβ and MAPK pathways. Prevention of oxidative stress can be also credited to the antioxidant potential of neferine which significantly prevented inflammation in the vascular endothelial cells induced by LPS. Therefore, neferine can be suggested as a potential drug for the treatment of vascular inflammatory linked ailments such as atherosclerosis and heart diseases.

4. Experimental

4.1. Materials

Neferine and LPS were obtained from Sigma Aldrich (St. Louis, MO, USA). Antibodies against iNOS, COX-2, pIkkα, pIkkβ, pIkβ, pJNK, pP38, pERK ½, tubulin, and horseradish peroxidase-conjugated (HRP) secondary antibodies were obtained from Santa Cruz Biotechnology, US. Penicillin (5000 U/mL), Streptomycin (5000 μg/mL), phosphate buffer saline (PBS), fetal bovine serum, bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT), assay kits, and western blot analysis kits were obtained from Sigma Aldrich (St. Louis, MO, USA). Primers and probes for mRNA gene expression tests were purchased from Life Technologies, CA, USA. All other chemicals were of analytical grade from Sigma Aldrich (St. Louis, MO, USA).

4.2. Cell culture

Human Umbilical Vein Endothelial Cells (HUVEC) and other endothelial cells including rat microglia were purchased from American Type Culture Collection (ATCC) (Manassas, US). Cells were cultured in 75 cm^2 flasks with M199 medium containing penicillin (100 U/mL), streptomycin (100 μg/mL), 10% fetal bovine serum, hydrocortisone, epidermal growth factor, with 5% carbon dioxide at 37 °C until reaching 90% confluence. The endothelial cells were incubated in 96-well plates for experiments with or without the presence of LPS and neferine at different concentrations. Addition of neferine was done 12 h prior to treatment with LPS (1 μg/mL).

4.3. Cell viability assay

Cell viability assay was conducted using MTT analysis following manufacturer's procedures. Cells were seeded in 96-well plates and treated with neferine for 24 h. The cells were then replaced with 100 μL fresh medium and 10 μL of MTT solution (5 mg/mL) for 4 h. DMSO was used to dissolve the formazan crystals and the absorbance was read using a microplate reader (Tecan Austria GmbH, Austria) at 570 nm with a reference of 670 nm wavelength. The results were presented as percentage mean±S.D. of at least three independent tests.

4.4. Nitric oxide (NO) production analysis

Endothelial cells were plated in 96-well plates, pretreated with neferine for 30 min and induced with LPS (1 μg/mL) for 24 h. Then the cell culture supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diamine in 5% phosphoric acid) at room temperature in the dark for 10 min. Sodium nitrite standard solution (0-100 μM) was used to determine the nitrite concentrations. The absorbance was read using a microplate reader (Tecan Austria GmbH, Austria) at 540 nm. The results were presented as μmol nitrite±S.D. of at least three independent tests.

4.5. TNF-α determination in endothelial cells (ELISA assay)

Endothelial cells were plated in 96-well plates, pretreated with neferine at different concentrations (5, 10, 25 μg/mL) for 30 min and induced with LPS (1 μg/mL) for 24 h. The level of TNF-α in culture supernatant of endothelial cells were assayed using ELISA kits (R&D Systems, Minneapolis, USA) following manufacturer's instructions. The results were presented as pg/mL supernatant±S.D. of at least three independent tests.

4.6. Gene expressions of pro-inflammatory cytokines

Endothelial cells were plated in 96-well plates, pretreated with neferine at different concentrations (5, 10, 25 μg/mL) for 30 min and induced with LPS (1 μg/mL) for 24 h. Total RNA were isolated from endothelial cells using TRIzol reagent (Life Technologies, CA, USA) and cDNA archive kit (Life Technologies, CA, USA) was used to synthesize cDNA. Quantitative real time polymerase chain reaction (qRT-PCR) was used to determine mRNA levels of TNF-α, IL-1β, iNOS, COX-2, and NADPH oxidase components with an ABI Prism 7500 Sequence Detector System (Life Technologies, CA, USA). TaqMan Universal PCR Master Mix Life Technologies, CA, USA) was used to perform the PCR reactions. Genes were quantified according to the internal control gene using a standard curve. The relative expression value of control group was set to 1. The results expressed were of at least three independent experiments.

4.7. Reactive oxygen species (ROS) determination

Endothelial cells were plated in 96-well plates, pretreated with neferine for 30 min and induced with LPS (1 μg/mL) for 4 h. Cells were incubated with 20 μM fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) at 37 °C for 45 min in the dark. Upon washing with PBS, the cells were then detected with DCFH-DA fluorometric detection fluorescent microplate reader at an excitation and emission wavelengths of 485 nm and 530 nm, for the determination of intracellular ROS levels. The intracellular ROS levels are directly proportional to the DCF fluorescence intensity. The results expressed were of at least three independent experiments.

4.8. Protein extraction and western blotting

Endothelial cells were plated in 96-well plates, pretreated with neferine at different concentrations (5, 10, 25 μg/mL) for 30 min and induced with LPS (1 μg/mL) at different time for each test. Cell lysis buffer containing protease and phosphatase inhibitors was added to the harvested cells. The total protein content was assayed using Lowry's method. The proteins were separated onto 12% SDS-polyacrylamide gel and then transferred on a nitrocellulose membrane, blocked with skimmed milk at room temperature for 1 h. The immunoblots were incubated with primary antibodies (iNOS, COX-2, pIkkα, pIkkβ, pIkβ-α, pJNK, pP38, pERK ½, α-tubulin) overnight at 4 °C. The immunoblots were then incubated with horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. The immunoblots were stained using enhanced chemiluminescence detection kit and subjected to detection using gel documentation system. The results expressed were of at least three independent experiments.

4.9. Statistical analysis

Results were analyzed using one-way analysis of variance (ANOVA-one way) followed by Dunnett's *post-hoc* analysis. All results are presented as mean±S.D. of at least three independent tests. P values less than 0.05 ($p < 0.05$) were regarded to statistical significance.

Conflicts of interest: None declared.

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