BOLETIN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
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Articulo Original / Original Article

Protective effect of trans-nerolidol on vascular endothelial cell injury induced by lipopolysaccharides

[Efectos protectores del trans-nerolidol en lesiones inducidas por lipopolisacáridos en células endoteliales vasculares]

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Section Biological activity

Received: 23 June 2023
Accepted: 15 August 2023
Accepted corrected: 11 November 2023
Published: 30 July 2024

Citation:
Yang N, Ma W.
Protective effect of trans-nerolidol on vascular endothelial cell injury induced by lipopolysaccharides
Bol Latinoam Caribe Plant Med Aromat
23 (4): 516 - 522 (2024).
https://doi.org/10.37360/blacpma.24.23.4.34

Abstract: This article aimed to discuss the protection of trans-nerolidol on vascular endothelial cells (ECs) injured by lipopolysaccharides. ECs were divided into four groups: normal, model, low and high dose trans-nerolidol treatment groups. The cell survival rate and the contents of NO in the cell culture supernatant were determined. The protein expression and transcript level of peroxisome proliferator-activated receptor-γ (PPARγ), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) were determined by western blotting and RT-PCR respectively. Compared with the normal group, cell livability, protein expression and mRNA transcript level of PPARγ and eNOS decreased, NO contents, protein expression and mRNA transcript tlevel of iNOS increased in model group significantly. Compared with model group, all the changes recovered in different degree in treatment groups. Hence, it was concluded that trans-nerolidol can alleviate the ECs injuryby the regulation of iNOS/eNOS through activating PPARγ in a dose-dependent manner.

Keywords: Vascular endothelial cells; Trans-nerolidol; Peroxisome proliferator-activated receptor-γ; Endothelial nitric oxide synthase; Inducible nitric oxide synthase

Resumen: Este artículo tiene como objetivo discutir la protección del trans-nerolidol en las células endoteliales vasculares (CE) dañadas por lipopolisacáridos. Las CE se dividieron en cuatro grupos: normal, modelo, grupos de tratamiento con trans-nerolidol de baja y alta dosis. Se determinó la tasa de supervivencia de las células y los contenidos de óxido nítrico (NO) en el sobrenadante del cultivo celular. La expresión de proteínas y el nivel de transcripción del receptor activado por proliferadores de peroxisomas-γ (PPARγ), el óxido nítrico sintetasa endotelial (eNOS) y el óxido nítrico sintetasa inducible (iNOS) se determinaron mediante western blot y RT-PCR, respectivamente. En comparación con el grupo normal, la viabilidad celular, la expresión de proteínas y el nivel de transcripción de PPARγ y eNOS disminuyeron, los contenidos de NO, la expresión de proteínas y el nivel de transcripción de iNOS aumentaron significativamente en el grupo modelo. En comparación con el grupo modelo, todos los cambios se recuperaron en diferentes grados en los grupos de tratamiento. Por lo tanto, se concluyó que el trans-nerolidol puede aliviar el daño en las CE regulando iNOS/eNOS a través de la activación de PPARγ de manera dependiente de la dosis.

Palabras clave: Células endoteliales vasculares; Trans-nerolidol; Receptor-γ peroxisoma proliferador-activado; Óxido nítrico sintetasa endotelial; Óxido nítrico sintetasa inducible

INTRODUCTION

Vascular endothelial cells (ECs) are flat cells that form the inner lining of major blood vessels. These cells help regulate blood flow (Bulut *et al*., 2022). Their smooth surface allows blood to flow smoothly. They also help regulate blood pressure and prevent coagulation (Sasaki *et al*., 2013). Due to its unique position in the vessel wall, ECs can feel hemodynamic changes and blood transmission signals through cell membrane receptors, and then deliver an immediate response by secreting substances in order to maintain the balance of blood vessels. Nitric oxide (NO) is a vasodilator. It is produced by the endothelial cells and causes the muscles of the vessel to relax (Siregar *et al.,* 2018). Also produced by endothelial cells is the vasoconstrictor endothelin (ET). ET and NO together constitute a balance system to participate in regulating vessel tension (Ihling *et al*., 2015). Synthesis of NO in ECs is catalyzed by nitric oxide synthase (NOS), which mainly includes two forms: endothelial NOS (eNOS) and inducible NOS (iNOS) (Liang *et al*., 2019). NOS utilizes tetrahydrobiopterin, NADPH, and molecular oxygen to convert l-arginine to l-citrulline and NO (Atkins *et al*., 2015). The massive release of NO can lead to endothelial damage, which results in vascular headaches such as migraine (Nowaczewska *et al*., 2020). Nerolidol (3,7,11-trimethyl-1,6,10-dodecatrien-3-ol), also known as peruviol, is a naturally occurring sesquiterpene alcohol present in the essential oil of various plants with a floral odor. Chemically, it exists in two geometric isomers, *trans* and *cis* forms. Nerolidol was found to exist as one of the bioactive compounds responsible for the biological activities demonstrated by the essential oil of source plants (Chan *et al.,* 2016). Recently our group studied the constituents of *Cinnamomum camphora* (Linn) Presl essential oil (CCEO) and their analgesic properties. An analgesia test was performed employing reserpine-induced migraine and nitroglycerin-induced migraine models. The present study showed that CCEOs have significant analgesic effects in experimental models of pain. The mechanism may be related to the action on NOS pathways (Yang *et al*., 2019; Fan *et al*., 2020). The majority of compound identified in CCEO was nerolidol (Yang *et al.,* 2019). The molecular mechanism of *trans*-nerolidol (TN) against ECs injury induced by lipopolysaccharide (LPS) was investigated in this study, which deals with the regulation of NO production and related protein and gene expression.

MATERIALS AND METHODS

Chemicals

The kits of NO (No. ml076492) were from Shanghai Enzyme-linked Biotechnology Co., Ltd. TRIzol (15596-026) was the product of Invitrogen of USA. The first-strand cDNA synthesis kits (RR036B) and One Step TB Green™ PrimeScript™ RT-PCR Kit II (SYBR Green) were from TaKaRa of Japan. 0.1% DEPC Water (KGDN4500), BCA protein quantitative detection kit (KGA902), SDS-PAGE gel preparation kit (KGP113), SDS-PAGE loading buffer (KGP101), Tris Buffer (KGP103X), Western Blotting detection kit (KGP1201), Cell Counting Kit-8(CCK-8, KGA317), developer and fixative (KGP116) and F-12K medium (KGM21127N-500) were from KeyGEN BioTECH of China. 96 well cell culture plates (3599) were from Corning Incorporated of USA. Peroxisome proliferators activated receptor–γ antibody (PPARγ, ab41928), eNOS antibody (ab300071), iNOS antibody (ab178945), and glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH, ab37168) were from Abcam Company. *Trans*-nerolidol (S315339) was bought from Shanghai Yuanye Biotechnology Co., Ltd. Its structure was fully characterized by chemical and spectroscopic methods. The purity was above 98.0% as determined by HPLC. The other reagents were analytical pure (Nanjing Chemical Plant). The used instruments mainly include super-clean worktable (SW-CJ-1FD, Suzhou Antai Airtech), invert microscope (OLYMPUS IX51), electrophoresis apparatus (Power Supplies Basic,), protein transfer system (Trans-Blot Turbo, Bio-rad), gel imaging system (SYNGENE G BOXChemiXR5), high-speed freezing centrifuge (ThermoSorvall ST 16R), microplate reader (MD Spectramac M3), real time-PCR system (Step one plus, ABI), Thermostatic oscillator (THZ-312, Shanghai Jinghong), electronic scale (JA3003, Suzhou Science).

Experimental grouping and cell culture

There were four groups: normal control group (I), LPS injury model group (II), *trans*-nerolidol low dosage protected group (III), and *trans*-nerolidol high dosage group (IV). Cells in the injury group were given 1 μg/mL LPS, while cells in the protection group were given 1 μg/mL LPS and 3 μM or 30 μM *trans*-nerolidol. Human Umbilical Vein Endothelial Cells (HUVEC-C) strain was from KeyGEN BioTECH. HUVEC-C cells were preserved in 90% F-12K medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator for 72 h.

The culture medium was changed every other day. The cell number was reset to 1 × 105/mL, and 160 μL cell suspensions were diverted to a 96-well cell culture plate. Each well of cell cultures was continuously incubated for 24 h. The test was done independently at least three times, and so it is in the later experiments.

Cell Proliferation and Viability Assay

The activity of *trans*-nerolidol on HUVEC was assessed by using the CCK-8 assay kit according to the producer's protocols. Mixing sterilized *trans*-nerolidol with complete medium resulting in concentration (3 μM, 30 μM) solution, then filtered that with a 0.22 μm filter membrane and stored at 4°C. Adding 100 μL of 5 x 10^4/mL HUWEC suspensions to the 96-well plate followed by incubating at 37°C till all the cells became adhesion, then 100 μL *trans*-nerolidol solution above was added. Added 10 μL CCK-8 solution per well and then incubated for 6 h before measuring its 450 nm absorbance. Cell survival rate (OD of treated cells/OD of normal cells) was calculated to evaluate the cell activity of the samples.

Nitric oxide generation assay

The cell supernatant NO levels were measured according to the kit instructions. The tested compounds and LPS were added to each well of cell cultures and incubated for 14 h, 100 μL fresh Griess reagent was added in the same volume of culture medium, and then the plates were shaken for 10 min for sufficient reaction. The absorbance was measured at 550 nm and the results were expressed as the difference between the NO productions.

Western blot assay of peroxisome proliferator-activated receptor-γ (PPARγ), eNOS, and iNOS protein levels

Cells were bred in 10 cm dishes. The culture plate was placed on ice and lightly washed twice with precooled PBS. Several reagents, including 10 μL phosphatase inhibitor, 1 μL protease inhibitor, and 5μL 100 mM phenyl methane sulfonyl fluoride, were added to 1 mL cold lysis buffer and then preserved in the ice for several minutes. After the *trans*-nerolidol treatment, the total protein was fully lysed in the lysis buffer. Samples were cultured on a rotator at 4℃ for 4 min repeatedly five times and centrifuged at 12,000 g for 5 min at 4℃. The supernatant protein concentration was determined by the bicinchoninic acid method, and twenty micrograms of protein were shipped onto a gel and subjected to SDS-PAGE gel electrophoresis. Electrophoretic products were electroblotted onto nitrocellulose membranes, which were sealed off with a blocking solution containing 5% defatted milk powder by shaking for 2 hr. Membranes were taken out and incubated with PPARγ, eNOS, and iNOS antibodies according to the manufacturer's protocol. Protein bands were imagined by enhanced chemiluminescence reagent with a kit. The apparent molecular masses of proteins were determined using electrophoresis color markers. G: BOX chemiXR5 supplied tools for processing protein band images. The grey density of bands was analyzed utilizing the Gel-Pro32 analyzer software, and the ratio of the optical density of target protein expression to that of GAPDH expression was computed to represent the relative expression amount.

Real-time quantitative polymerase chain reaction (RQ-PCR) analysis of PPARγ, eNOS, and iNOS mRNA

Total RNA was collected with Trizol, and the A260/A280 value of the preparation was in the range of 1.8-2.1. Each sample (5 μg) was taken to manufacture cDNA by reverse transcription. The PCR primers were planned as follows: PPARγ, Forward: GCTGAATCCAGAGTCCGCTGAC, Reverse: TCGCCCTCGCCTTTGCTTTG, 98bp; eNOS, Forward: CGGCATCACCAGGAAGAAGACC, Reverse: CCTTCACTCGCTTCGCCATCAC, 95bp; iNOS Forward: GTGCGTTACTCCACCAACAA, Reverse: CGGAAGTCGTGCTTGCCATCA, 84 bp; GAPDH, Forward: AGATCATCAGCAATGCCTCCT, Reverse: TGAGTCCTTCCACGATACCAA, 90 bp. The PCR reaction was undertaken in a 20 μL reaction volume. The final concentrations of reactants were as follows: 2 μL forward primer, 10 μM; 2 μL reverse primer, 10 μM; PCR Master Mix (SYBR Green) 10 μL; ddH_2O 7 μL. PCR amplification was carried out using GAPDH primers as the internal standard. The relative level of the transcript of the target gene was determined.

Statistical analysis

Data were presented as mean values ± SD. The significance of differences between the means among different groups was analyzed by one-way ANOVA with SPSS13.0 and the level of $p<0.05$ or $p<0.01$ was taken as statistically significant.

RESULTS

To assess the effects of LPS and *trans*-nerolidol on

the cell viability, we used the CCK-8 assay, HUWEC-C cells were pretreated with various concentrations (3 and 30 μM) of TRANS-NEROLIDOL for 12 h, and then unmasked to 1 μg/mL LPS for 6 h. Compared with the control group, the viable cells in the LPS-exposed group were significantly decreased to 52.76 ± 2.8% (p<0.01). However, after the pretreatment with *trans*-nerolidol, the cell viability was 83.43 ± 4.04% and 92.11 ± 5.28%, respectively. A significant increase emerged, compared with the non-pretreatment group (p<0.01) (Figure No. 1).

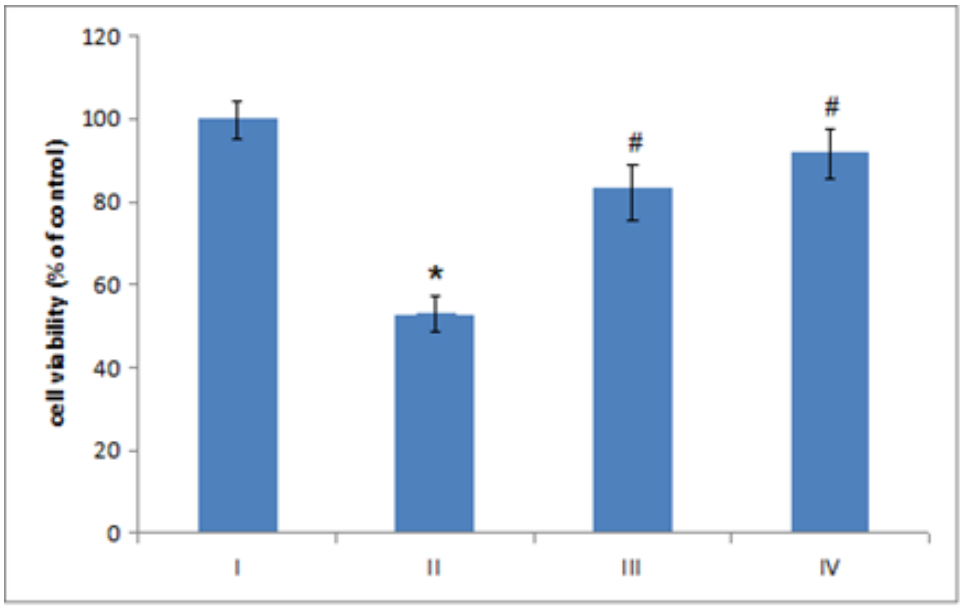

Figure No. 1
Detection of cell viability. I, Normal group; II, injury model group; III, Trans-nerolidol low dosage group; IV, Trans-nerolidol high dosage group
(*Compared with that of the normal group, p<0.01; #Compared with that of the model group, p<0.01)

The NO production of ECs could be regarded as signs of the activation of those inflammatory cells. The effects of *trans*-nerolidol treatment on LPS-induced changes in NO levels are shown in Figure No. 2. *trans*-nerolidol could reduce the generation and the release of NO from HUVEC-C induced by LPS. The NO production ((34.734 ± 0.104) μmol/mL) in the LPS stimulation group was markedly increased compared with that ((9.847 ± 0.226) μmol/mL) of the normal control group, while *trans*-nerolidol could significantly lower the content increase induced by LPS at two concentration levels, and the NO production after the administration of TRANS-NEROLIDOL was decreased to (18.881 ± 0.917) and (15.619 ± 1.540) μmol/mL, respectively.

For the attempt to understand the molecular mechanisms that underlie the antiinflammatory activity of *trans*-nerolidol, the expression level of PPARγ, eNOS, and iNOS protein was investigated in the *trans*-nerolidol pretreated HUVEC-C under exposure to LPS in western blotting assay, the expression level of PPARγ, eNOS protein waned as cells were exposed to 1 μg/mL LPS for 6 h. However, the pretreatment of *trans*-nerolidol could attenuate the downregulation of the PPARγ, eNOS protein to counteract ECs damage. *Trans*-nerolidol could reduce the increased expression level of iNOS protein as cells were exposed to 1 μg/mL LPS for 6 h (p<0.01) (Figure No. 3). The relative level of the transcript of the target gene was accurately calculated. The levels of the PPARγ and eNOS transcripts in the injury group were lower than those of the controls, but their decrease was significantly raised by *trans*-nerolidol in a dose-dependent manner (p<0.01). *Trans*-nerolidol could reduce the increased transcript level of iNOS induced by LPS (Figure No. 4), corresponding to a similar trend of the protein levels beheld in the Western blot analysis (Figure No. 3).

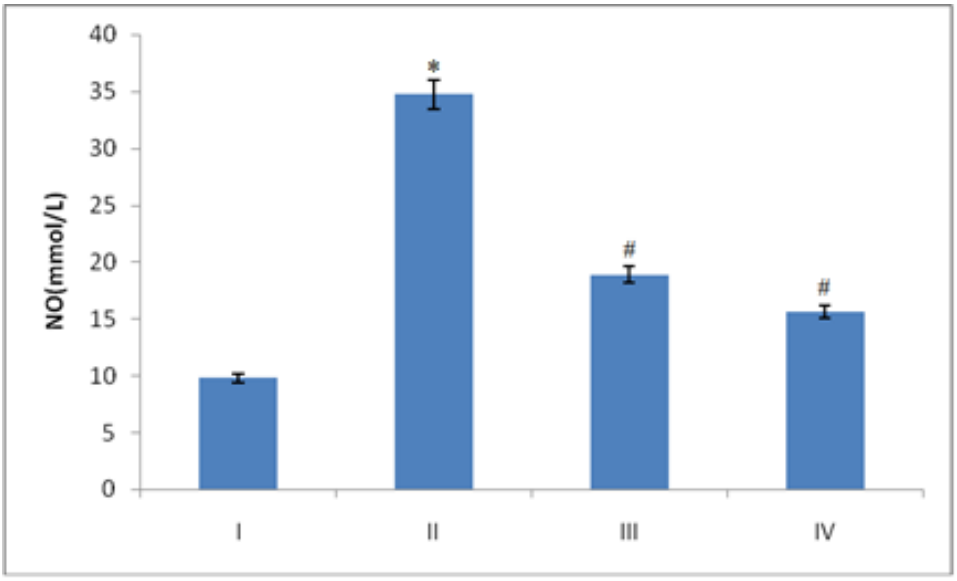

Figure No. 2
Effect of *trans*-nerolidol on NO contents in HUVEC-C
I, Normal group; II, injury group; III, *trans*-nerolidol low dosage group; IV, *trans*-nerolidol high dosage group. (*Compared with that of the normal group, $p<0.01$; #Compared with that of the model group, $p<0.01$)

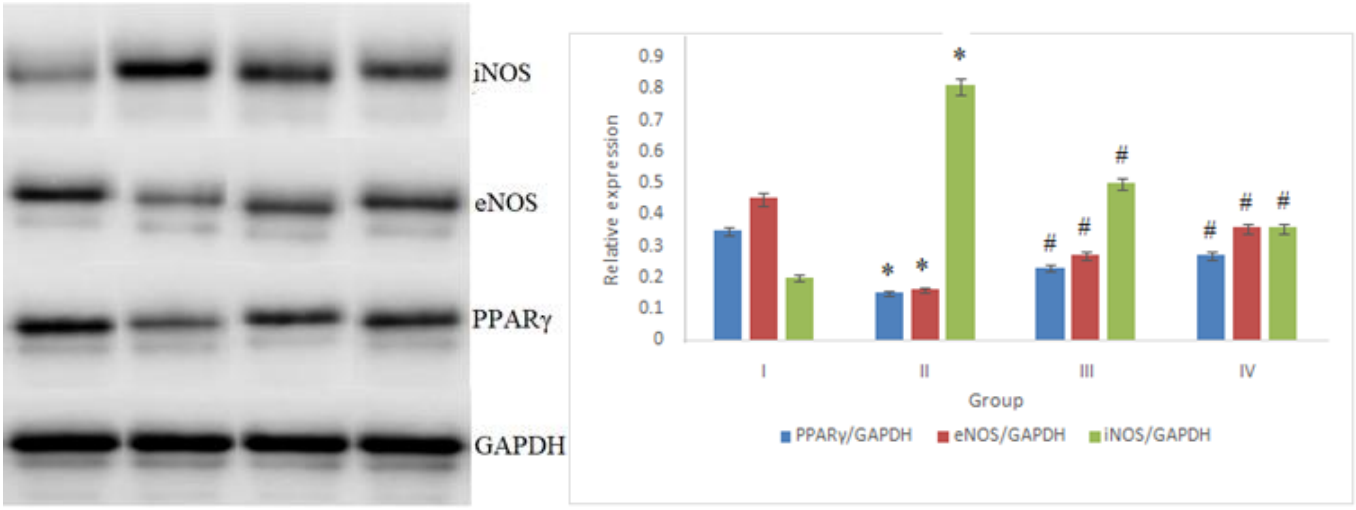

Figure No. 3
Effect of *trans*-nerolidol on expression of the PPARγ, eNOS and iNOS (bottom) protein, respectively.
I, Normal group; II, injury group; III, *trans*-nerolidol low dosage group; IV, *trans*-nerolidol high dosage group. (*Compared with that of the normal group, $p<0.01$; #Compared with that of the model group, $p<0.01$)

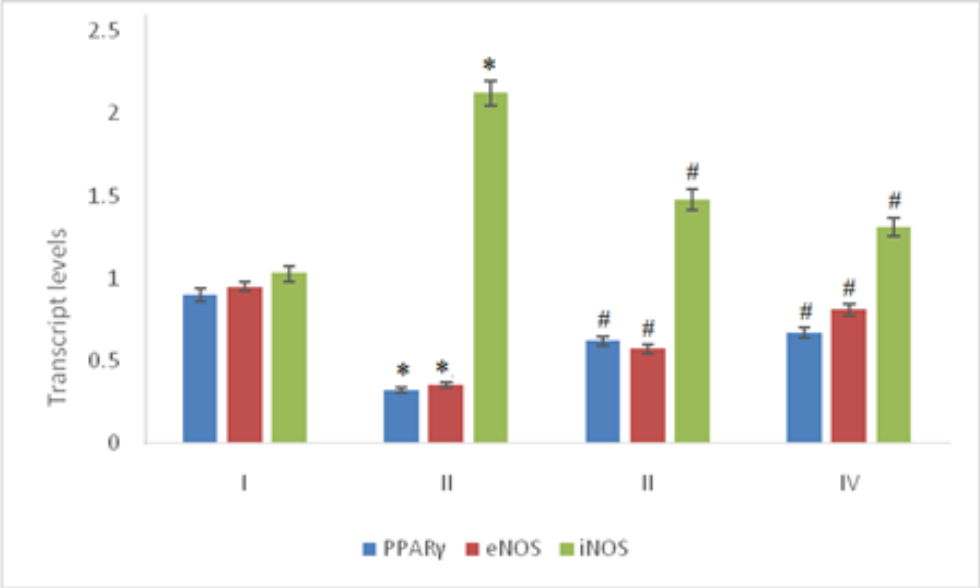

Figure No. 4
Effect of *trans*-nerolidol on transcript levels in HUVEC-C
I, Normal group; II, injury group; III, *trans*-nerolidol low dosage group; IV, *trans*-nerolidol high dosage group. (*Compared with that of the normal group, $p<0.01$; #Compared with that of the model group, $p<0.01$)

DISCUSSION

ECs have powerful effects on the tone of vascular smooth muscle, causing either contraction or relaxation (Yan *et al*., 2013). The basic release of NO can protect the cerebral vascular endothelium and maintain cerebral hemodynamic stability. NO causes the muscles of the vessel to relax (Liu *et al.*, 2022). One factor that triggers the production of NO is physical activity. Vascular dilation is important during activity to keep blood pressure from rising too much (Daneva *et al*., 2021). A second function of NO is to keep platelets from sticking together in the vessels. Damage to the vascular endothelial cells causing a reduction of NO is a risk factor for atherosclerosis (Gliozzi *et al*., *20*19). Under the physiologic condition eNOS was predominantly expressed in ECs, while iNOS was almost undetectable (Tajadura *et al*., 2020). In the pathologic state iNOS was substantially expressed to produce excess NO (Wilmes *et al*., 2020). The injury of ECs can lead to endothelial dysfunction. PPARγ is a member of the nuclear hormone receptor superfamily of transcription factors that regulate genes involved in lipid and glucose metabolism. PPARγ is expressed in smooth muscle and endothelial cells of the vascular wall and exerts pleiotropic effects on metabolism and inflammation in vascular biology (Blanquicett *et al*., 2010). PPARγ assists with the expression of eNOS (Guo *et al*., 2020), and PPARγ can also decrease the expression of iNOS by reducing the MAPK/NF-κB signaling (Abdel-Wahab *et al*., 2022).

CONCLUSION

In this study, *trans*-nerolidol treatment could significantly lower the level of iNOS, and increase the amount of eNOS, altogether reducing the content of NO for the LPS-inducing damage of HUVEC-C. It effectively improved endothelial dysfunction and inflammatory response by the balance regulation of iNOS/eNOS through activating PPARγ. This study revealed the therapeutic role of *trans*-nerolidol in remedying vascular endothelial cell dysfunction in the *in-vitro* experimental research.

Ethics approval

This study was conducted with the permission of the Ethics Review Committees of Nanjing University of Chinese Medicine of China.

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