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Biao Zhang¹, Junkai Zhao², Jiamin Yin¹, Jiaping Chen², and Lingqi Xu²

¹Department of Central Laboratory, Suzhou Hospital of Integrated Traditional Chinese and Western Medicine, Suzhou, China; ²Department of Emergency, Suzhou Hospital of Integrated Traditional Chinese and Western Medicine, Suzhou, China.

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Abstract

The study aims to investigate the effect of ginsenoside Rb1 on improving neutrophil extracellular traps-induced vascular endothelial damage. The CCK8 assay was used to evaluate the impact of ginsenoside Rb1 on cell activity. Flow cytometry was used to assess cell apoptosis. E-cadherin was used to determine the damage to endothelial cells, and caspase 3 was used to detect apoptosis-related proteins. Ginsenoside Rb1 (100 $\mu\text{mol/L}$) could significantly increase the cell viability ($p < 0.05$). At this concentration, the damage caused by neutrophil extracellular traps to human umbilical vein endothelial cells could be reversed ($p < 0.05$). Ginsenoside Rb1 (100 $\mu\text{mol/L}$) could significantly inhibit the apoptosis and the expression of caspase3 protein caused by neutrophil extracellular traps ($p < 0.05$). Ginsenoside Rb1 can reduce vascular endothelial injury induced by neutrophil extracellular traps, ginsenoside Rb1 protects vascular endothelium by inhibiting cell apoptosis.

Introduction

Neutrophil extracellular traps, web-like structures released by activated neutrophils and composed of DNA, proteins, and granular components, play a crucial role in innate immunity by trapping and killing pathogens (Wang et al., 2024). However, under pathological conditions, the abnormal formation and accumulation of neutrophil extracellular traps can give rise to tissue damage and inflammation, worsening the progression of diseases. Neutrophil extracellular traps can cause vascular endothelial damage (Zhu et al., 2024). The DNA fibers and granular proteins within neutrophil extracellular traps can directly interact with vascular endothelial cells, leading to the disruption of cell membranes and impairment of cell structures (Yang et al., 2023). Neutrophil extracellular traps can release reactive oxygen species (ROS), which induces oxidative stress in vascular endothelial cells, damages the cell

membranes, and causes vascular endothelial dysfunction (Zhou et al., 2024). The DNA in neutrophil extracellular traps can bind to toll-like receptors on vascular endothelial cells, activating them, promoting inflammatory responses, and ultimately leading to cell death (Blanco et al., 2021).

Neutrophil extracellular traps induce endothelial cell apoptosis via multiple routes, such as DNA damage, oxidative stress, inflammatory responses, and the effects of cytokines, among others (Carmona-Rivera et al., 2015). These mechanisms are interrelated and collaboratively facilitate the advancement of endothelial cell apoptosis. Neutrophil extracellular traps cause DNA damage in vascular endothelial cells, resulting in cell cycle arrest and activating apoptotic signals (Yu et al., 2022). Additionally, DNA damage can also give rise to genomic instability, further promoting cell apoptosis. Neutrophil extracellular traps release reactive oxygen



species, triggering oxidative stress in endothelial cells, damaging cell membranes and proteins, and leading to cell apoptosis. Therapeutic strategies that inhibit cell apoptosis might offer novel therapeutic directions for related diseases (Guo et al., 2024).

Ginsenoside Rb1 is capable of inhibiting apoptosis in multiple cell types. Research indicates that ginsenoside Rb1 mitigates myocardial cell apoptosis induced by myocardial ischemia-reperfusion injury by activating the mTOR signaling pathway, thereby enhancing cardiac function and reducing the release of myocardial enzymes (Qin et al., 2021). Ginsenoside Rb1 acts as a potential natural glutathione reductase agonist and can prevent H9C2 cell apoptosis induced by oxidative stress by regulating apoptosis related to the BCL-2 family (Zhang et al., 2017). Simultaneously, ginsenoside Rb1 exhibits a protective effect on vascular endothelium.

Studies have discovered that ginsenoside Rb1 safeguards human umbilical vein endothelial cells from high glucose-induced mitochondrial-related apoptosis by activating the SIRT3 signaling pathway (Ke et al., 2021). Ginsenoside Rb1 demonstrates remarkable anti-apoptotic effects in different cell types and disease models. Nevertheless, it remains undetermined whether ginsenoside Rb1 can ameliorate vascular damage caused by neutrophil extracellular traps by inhibiting apoptosis. This project aims to explore the mechanism of ginsenoside Rb1 to improve vascular damage induced by neutrophil extracellular traps and furnish a new target to treat vascular endothelial injury.

Materials and Methods

Cell culture

In this study, human venous endothelial cells were purchased from iCell and maintained in RPMI 1640

(Gibco, C11875500BT) media containing 10% fetal bovine serum (BDBIO, F801-050Hi). Human primary neutrophils were isolated using the neutrophil isolation kit (Solarbio, P9040). Human primary neutrophils were resuspended in DMEM supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin solution at a concentration of 1×10^6 cells/mL. All cells were grown at 37°C in the presence of 5% CO₂.

Neutrophil extracellular traps

Freshly isolated human or murine neutrophils were plated in 6-well culture plates (4×10^6 cells/mL) and stimulated with 100 nmol/L phorbol 12-myristate 13-acetate (PMA). To remove the cells, the culture medium was carefully removed from each well, and it was then washed twice with cold phosphate-buffered saline, which contained the pellet that contained neutrophil extracellular traps. Isolated neutrophil extracellular traps were then stored at -80°C before being used (Xie et al., 2023).

CCK8

The human venous endothelial cell viability was assessed through the CCK8 assay (Cai et al., 2024). A 96-well plate was prepared with 100 µL of cell suspension (1,000 cells/well) and incubated in a cell culture incubator at 37°C with 5% CO₂ overnight. The culture medium was aspirated, and wells were treated with varying concentrations of the drug-containing medium (final concentrations of 1, 5, 10, 50, 100, 200, and 300 µmol/L) in respective experimental wells. The control group was treated with an equal volume of medium containing PBS at a concentration not exceeding one thousandth. Cells were then incubated for an additional 24 hours. After proper incubation in the 96-well plate, the culture medium was aspirated, and 10 µL CCK8 (Medicalbio, CCK-04) solution was added to the

Box 1: Immunofluorescence detection

Principle

The expression of E-cadherin in human venous endothelial cells was examined through immunofluorescence assay.

Requirements

24-well plates (Servicebio, CCP-24H), PBS (Servicebio, G4202), BSA (Servicebio, GC305010), 4% paraformaldehyde (Servicebio, G1101), E-cadherin antibodies (Servicebio, GB12083), Cy3 conjugated goat anti-mouse IgG (H+L)(Servicebio, GB21301), PBST (Servicebio, G2157), DAPI(Servicebio, G1012), Fluorescence microscopy (Olympus IX73, Japan).

Procedure

Step 1: Cells were seeded in 24-well plates at a density of 1×10^4 cells/dish.

Step 2: Following PBS rinsing, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and washed

thrice with PBS.

Step 3: Blocking was carried out with 3% BSA at room temperature for 30 min.

Step 4: Primary antibodies (E-cadherin, 1:1000) were incubated overnight at 4°C, followed by three washes with PBS.

Step 5: Cells were then incubated in 3% BSA in PBST for 1 hour in the dark with the respective fluorescent-conjugated secondary antibody (Cy3 conjugated goat anti-mouse IgG (H+L), GB21301, 1:500) followed by washing with PBST.

Step 6: DAPI was applied to the nuclei for 5 min, washed with PBS, and observed by fluorescence microscopy.

Step 7: The fluorescence intensity was analyzed using ImageJ software.

Reference

Xie et al., 2023

corresponding experimental wells, followed by an incubator for 1 hour. Subsequently, the absorbance of each well was measured using an enzyme labeling instrument set at a wavelength of 450 nm.

Cell apoptosis analysis

Cell apoptosis was determined using an annexin V-FITC/PI apoptosis kit [MultiSciences Biotech Co. AP101]. The human venous endothelial cells were digested by trypsinization without EDTA via centrifugation at $300 \times g$ for 5 min at 4°C . The cells were washed with pre-cooled PBS two times and resuspended with 1x binding buffer. Annexin V (5 μL) and PI (10 μL) were added to the cell suspension. They were incubated for 5 min away from light and detected by Beckman flow cytometry (USA) (Leon et al., 2024).

Western blotting

The human venous endothelial cells sample was lysed in RIPA buffer containing 1 mM PMSF, protease inhibitors, and phosphatase inhibitors (Beyotime Biotechnology) for 30 min. A protein sample was then determined using the BCA method (Beyotime Biotechnology). Using 10% SDS-PAGE electrophoresis, gels were transferred to PVDF (0.45 μm) membranes. PVDF membranes were blocked in 5% fat-free milk for 60 min. PVDF membranes were then rinsed with TBST buffer 3 times for 5 min and probed with BAX (Biodragon, PB4259, 1:1000), caspase 3 (Zen-bio, R23315, 1:1000), Bcl-2 (Proteintech, 60178-1-Ig, 1:3000), anti-GAPDH (Biodragon, B1034, 1:5000) at 4°C overnight. After rinsing

with TBST buffer 3 times for 5 min, PVDF membranes were then incubated in HRP-conjugated secondary antibody (ABclonal, AS014, 1:7500) or multi-rAb HRP-goat anti-mouse recombinant secondary antibody (H+L) (Proteintech, RGAM001, 1:7500) at room temperature for 1 hour. After rinsing with TBST buffer 3 times for 5 min, the membrane was imaged at a Tanon scan imager after ECL (Medicalbio, PT01001) reagent visualization, all bands were analyzed by Image J software (Hoste et al., 2019).

Statistical analysis

The data were expressed as the mean and standard error of the mean (SEM). The analysis was performed by Graphpad Prism 5 (Graphpad Software, USA). We used a Student's t-test to determine whether the two groups differed statistically. P-values less than 0.05 were considered significant.

Results

Cell viability

The CCK8 experiment shows that when the concentration of Rb1 is less than 100 $\mu\text{mol/L}$, there is no cytotoxicity to human umbilical vein endothelial cells (Figure 1 A). After adding neutrophil extracellular traps, the cell viability decreases. Rb1 at a concentration of 100 $\mu\text{mol/L}$ can significantly increase cell viability (Figure 1 B).

Vascular endothelium

Employing E-cadherin to assess the degree of vascular endothelial injury, the study revealed that after the addition of neutrophil extracellular traps, the vascular endothelium was damaged. However, after adding ginsenoside Rb1 at a concentration of 100 $\mu\text{mol/L}$, the damage caused by neutrophil extracellular traps to human venous endothelial cells could be reversed (Figure 2 A-B).

Human venous endothelial cell apoptosis

Flow cytometry was used to analyze the apoptosis of cells. The study found that after adding neutrophil extracellular traps, the apoptosis of cells increased significantly (Figure 3 A-B). Different concentrations of ginsenoside Rb1 were added for intervention, and it was found that Rb1 at a concentration of 100 $\mu\text{mol/L}$ could significantly inhibit the apoptosis of cells caused by neutrophil extracellular traps (Figure 3 C-D).

Expression of apoptosis-related proteins

Neutrophil extracellular traps can significantly up-regulate the expression of apoptosis-related proteins caspase 3, and after adding ginsenoside Rb1, the expression of apoptosis-related proteins caspase 3 is down-regulated (Figure 4 A-B).

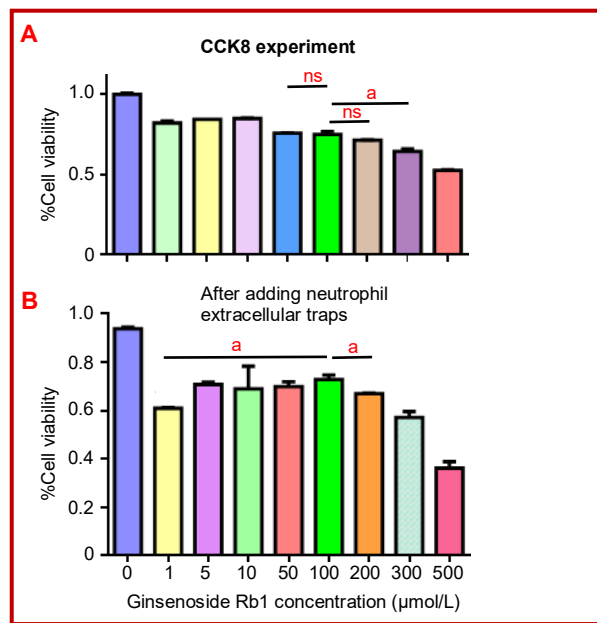


Figure 1: Effects of ginsenoside Rb1 on the cell viability of human umbilical vein endothelial cells using the CCK8 experiment (A) and after adding neutrophil extracellular traps (B). Data are mean \pm SEM; n=3; Compared to 100 $\mu\text{mol/L}$, ns = not significant, *p<0.05

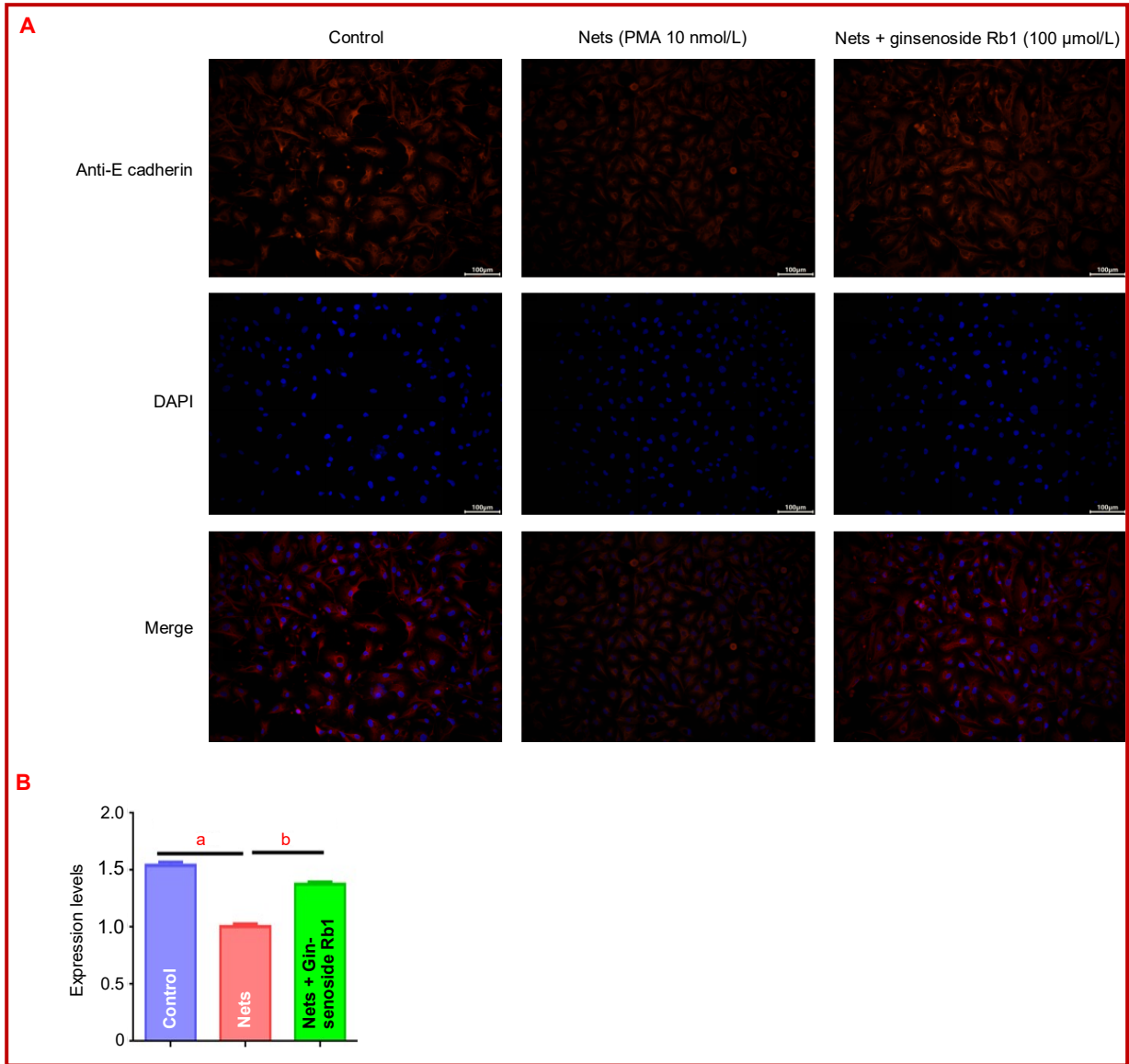


Figure 2: Effect of ginsenoside Rb1 (100 μ mol/L) on the damage to vascular endothelial cells (A). E-cadherin was used to evaluate the injury. Expression levels after using ginsenoside Rb1. Data are mean \pm SEM; n=3; Compared to the control group ^ap<0.05, Compared to the NETs group ^bp<0.05

Discussion

This study found that ginsenoside Rb1 significantly attenuated the damage of neutrophil extracellular traps to vascular endothelial cells by inhibiting apoptosis. CCK8 experiment reveals that in contrast to the neutrophil extracellular traps group, the supplementation of 100 μ mol/L ginsenoside Rb1 can markedly enhance cell viability. Immunofluorescence results show that ginsenoside Rb1 can up-regulate the expression of E-cadherin and protect vascular endothelial cells. Flow cytometry analysis shows that 100 μ mol/L of Rb1 can significantly inhibit the apoptosis caused by neutrophil extracellular traps. Ginsenoside Rb1 could significantly downregulate the expression of caspase-3

Existing studies have confirmed that ginsenoside Rb1 has the effect of protecting vascular endothelium. Previous studies have not involved the effect of neutrophil extracellular traps on vascular endothelial injury. Instead, they have mostly focused on vascular endothelial injury caused by other physical and chemical factors. One study has demonstrated that ginsenoside Rb1 can alleviate TNF- α -induced oxidative stress, inflammation, and apoptosis in human venous endothelial cells by inhibiting the NF- κ B, JNK, and p38 signaling pathways (Zhou et al., 2017). Another study has revealed that ginsenoside Rb1 protects human venous endothelial cells from high glucose-induced mitochondrial-related apoptosis by activating the SIRT3 signaling pathway (Ke et al., 2021). Ginsenoside Rb1

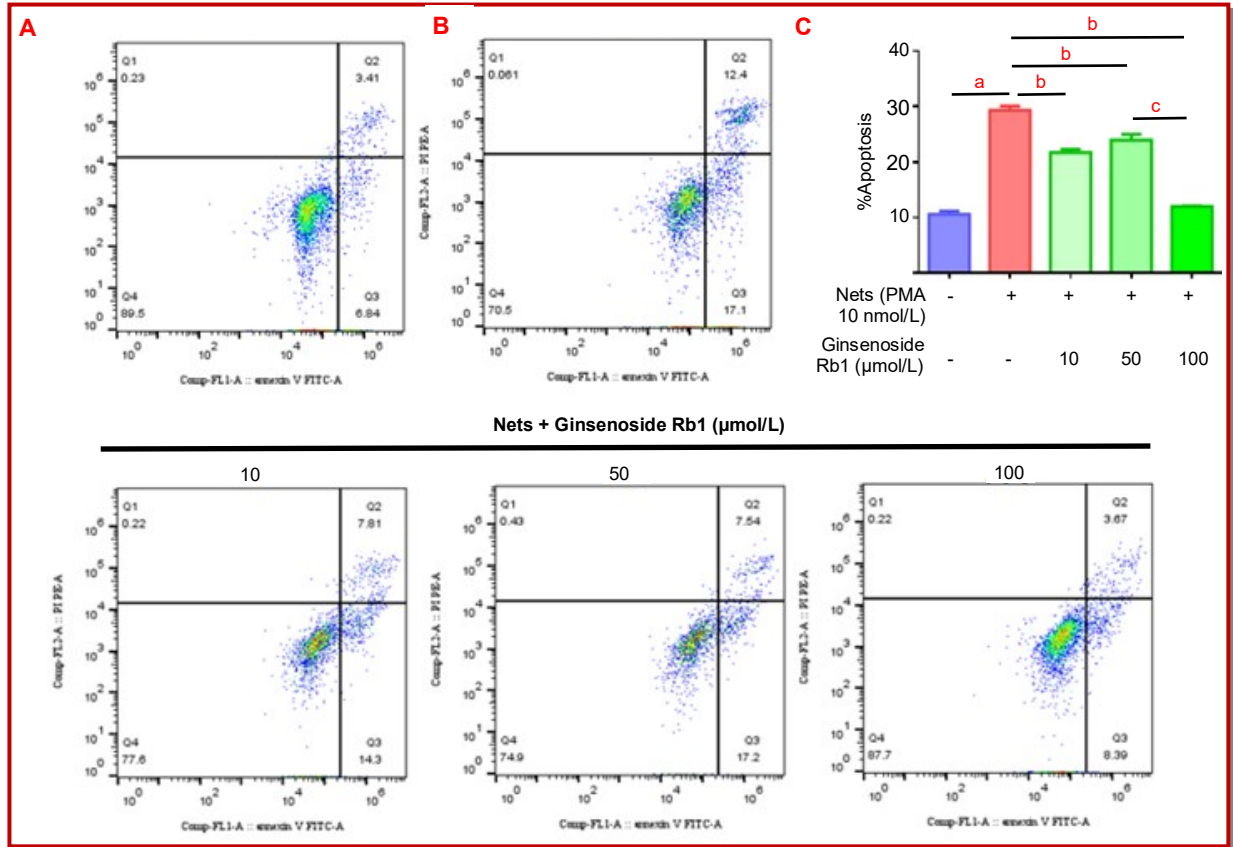


Figure 3: Effects of ginsenoside Rb1 on human umbilical vein endothelial cells apoptosis. Flow cytometry analyzed cell apoptosis. Adding neutrophil extracellular traps significantly increased cell apoptosis (A, B). Different concentrations of Rb1 were used for intervention, and 100 μmol/L Rb1 significantly inhibited the apoptosis caused by neutrophil extracellular traps (C, D). Data are mean ± SEM; n=3; Compared to the control group, ^ap<0.05; Compared to the NETs group, ^bp<0.05; Compared to ginsenoside Rb1 (50 μmol/L) group, ^cp<0.05

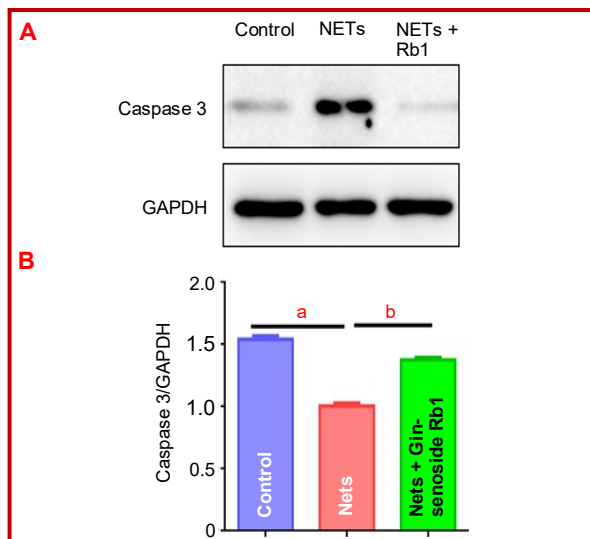


Figure 4: Effects of ginsenoside Rb1 on the expression of caspase 3 proteins. Neutrophil extracellular traps significantly up-regulate the expression of caspase 3, an apoptosis-related protein, while ginsenoside Rb1 addition significantly down-regulates its expression. Data are mean ± SEM. Compared to the NETs (PMA 10 μmol/L), ^ap<0.05; compared Nets with ginsenoside Rb1, ^bp<0.05

can mitigate H₂O₂-induced oxidative damage in human venous endothelial cells (Zheng et al., 2020), encompassing reducing apoptosis, oxidative stress, and the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 proteins. This study aims to thoroughly investigate the protective effect of ginsenoside Rb1 on vascular endothelial injury induced by neutrophil extracellular traps. This research perspective is more closely related to the pathophysiological characteristics of acute lung injury, which helps in accurately elucidate the underlying pathophysiological mechanisms during acute lung injury and provides a more targeted theoretical basis for subsequent related research and clinical practice.

The results of this study distinctly manifest the positive role of ginsenoside Rb1 in counteracting the damage to human umbilical vein endothelial cells induced by neutrophil extracellular traps, covering multiple aspects including cell viability, vascular endothelial injury, cell apoptosis, and the expression of apoptosis-related proteins. This provides copious information for an in-depth comprehension of the protective mechanism of ginsenoside Rb1. E-cadherin, as a crucial molecule for maintaining the integrity of intercellular connections among

vascular endothelial cells, when damaged under the action of neutrophil extracellular traps, implies the impairment of the vascular endothelial barrier function. This might further trigger a series of pathological alterations such as enhanced vascular permeability and infiltration of inflammatory cells (Chen et al., 2024). Nevertheless, 100 $\mu\text{mol/L}$ of ginsenoside Rb1 can reverse the damage of neutrophil extracellular traps to the vascular endothelium. Flow cytometry analysis results reveal that neutrophil extracellular traps can significantly induce apoptosis of human umbilical vein endothelial cells. In the intervention experiments with varying concentrations of ginsenoside Rb1, 100 $\mu\text{mol/L}$ of ginsenoside Rb1 can significantly suppress the apoptosis of cells induced by neutrophil extracellular traps. This suggests that ginsenoside Rb1 plays a pivotal role in regulating cell apoptosis, which might be accomplished through multiple pathways. The study has identified that neutrophil extracellular traps can significantly up-regulate the expression of the apoptosis-related protein caspase 3. This coincides with the result of neutrophil extracellular traps-induced cell apoptosis and further validates the critical role of caspase 3 in the process of neutrophil extracellular traps-mediated cell apoptosis. Ginsenoside Rb1 can significantly down-regulate the expression of caspase 3, indicating that the inhibitory effect of ginsenoside Rb1 on cell apoptosis is at least partly achieved through the regulation of caspase 3.

However, this study has several limitations. Firstly, in light of the restricted research conditions, neither animal models nor clinical trials were utilized to verify the research results. Secondly, cell death is an intricate process, and additional experiments may be necessary to confirm this conclusion. Finally, the specific mechanism through which ginsenoside Rb1 modulates cell apoptosis via caspase 3 requires further in-depth exploration.

Conclusion

Ginsenoside Rb1 has a notable protective effect on neutrophil extracellular traps-induced vascular endothelial injury, and its mechanism might be associated with the inhibition of apoptosis effects.

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Ethical Issue

The study protocol has been approved by the Medical Ethics

Committee of Suzhou Hospital of Integrated Traditional Chinese and Western Medicine (No. 2024002; Date: 2024-3-5).

Conflict of Interest

Authors declare no conflict of interest

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Author Info

Lingqi Xu, Jiaping Chen (Principal contact)

e-mail: xu.ling.qi@163.com; cjpszmu@126.com

First three authors contributed equally