

Vaccarin attenuates the human EA.hy926 endothelial cell oxidative stress injury through inhibition of Notch signaling

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Abstract. Endothelial cell injury is an essential component of atherosclerosis and hypertension. Atherosclerosis and other macrovascular diseases are the most common complications of diabetes. Vaccarin is a major flavonoid glycoside in *Vaccariae semen*, and is expected to be useful in the treatment of vascular diseases. The aim of the present study was to evaluate the possible effects of vaccarin in human umbilical vein endothelial cells (EA.hy926) induced by hydrogen peroxide (H₂O₂) and its underlying mechanism in the prevention and treatment of H₂O₂ injury. In this study, the EA.hy926 cells were exposed to 250, 500 and 1000 μ M H₂O₂ for 2 and 4 h in the absence or presence of vaccarin, and the cell injury induced by H₂O₂ was examined via SRB. Cell migratory ability, lactate dehydrogenase (LDH) leakage, malondialdehyde (MDA) levels and decreasing superoxide dismutase (SOD) activity were evaluated by the wound healing assay and corresponding assay kits. Cell apoptosis was detected by flow cytometry with Annexin V-fluorescein isothiocyanate/propidium iodide Apoptosis Detection kit and Hoechst staining. Furthermore, western blot detected the protein expressions of Notch1, Hes1 and caspase-3. Following treatment with H₂O₂, it was found that H₂O₂ stimulated cell injury in a dose-dependent manner, including reducing cell viability and cell migratory ability, increasing LDH leakage and MDA levels, and decreasing SOD activity. H₂O₂ further accelerated cell apoptosis via activation of Notch1 and the downstream molecule Hes1. Preincubation with vaccarin was found to protect EA.hy926 cells from H₂O₂-induced cell oxidative stress injury, which promoted cell viability and cell migratory ability, inhibited the level of LDH and MDA, but enhanced the activity of SOD. In particular, in addition to downregulation Notch signaling, vaccarin treatments also downregulated caspase-3, a cell apoptotic pathway-related

protein. These findings indicated that vaccarin may be able to selectively protect vascular endothelium from dysfunction induced by H₂O₂.

Introduction

Macrovascular diseases, such as atherosclerosis, are the most frequent complications of diabetes (1). Diabetes mellitus impairs endothelial function and may be an indicator of the cardiovascular disease development (2,3). Vascular endothelium is considered to play an essential role in diabetes-associated vascular dysfunction, including atherosclerosis (4). The endothelium plays a significant role in the regulation of vascular function and the development of physiological and pathophysiological inflammation (5-8). Endothelial cell injury is a critical element of atherosclerosis and hypertension (9,10). Previous studies have shown that H₂O₂ induced endothelial cell apoptosis, and causes cellular dysfunction and cell death (11,12). The vascular function pathogenesis is complicated and there are a number of signaling pathways, including the Notch pathway.

The Notch signaling pathway is one of the pathways that plays a significant role in cell differentiation, primarily determining and regulating cell survival (13,14). In mammals, four receptors (Notch1-Notch4) and five ligands, including Jagged1, Jagged2, Delta-like 1 (Dll1), Dll3 and Dll4, have been discovered (15,16). Notch signaling also affects cellular activities, including proliferation, migration, growth, differentiation and death (17). In addition, Notch activity controls the communication between cells, signal transduction in the cytoplasm and gene transcription in the nucleus. The genes downstream of Notch signaling include Hairy and enhancer of split 1 (Hes1) and the Hairy-related transcription (HRT) factor family. The binding of a ligand and receptor induces a conformational change of the Notch receptor. This allows an extracellular metalloprotease to cleave the receptor, which allows the γ -secretase-mediated protease to release the Notch intracellular domain. Subsequently, the Notch intracellular domain travels into the nucleus where it activates the transcription of downstream genes, such as Hes1 (18). A recent study indicates that in H₂O₂-induced cell apoptosis, the Notch signaling pathway was upregulated (19), indicating that Notch inhibition may be a useful method in the protection of cells from H₂O₂-induced apoptosis.

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Thus far, detrimental effects induced by H_2O_2 on human endothelial cells can be suppressed by numerous types of plant active substances (12,20-21). *Vaccariae semen*, the seeds of *Vaccaria segetalis* (Neck.) Garcker. ex Asch. (Caryophyllaceae), is a famous traditional medicinal plant (22) for activating blood to promote menstruation, invigorating blood circulation, regulating menstrual disturbance and dispelling edema, promoting diuresis and milk secretion, and relieving carbuncles (23-24). It contains flavonoids, cyclic peptides, triterpene saponins, lipids, aliphatic acids, monosaccharides, biotin, and coumarin (25-31), with a few of these compounds demonstrating bioactivity, such as anti-angiogenesis and growth-inhibitory activity on luteal, HL-60 and endothelial cells (32-33). Vaccarin is a major flavonoid glycoside in *Vaccariae semen* and is considered one of the main active constituents, which has attracted increasing attention (34). The present study investigated the protective effect of vaccarin (Fig. 1) on human umbilical vein endothelial cell lines (EA.hy926) injured by H_2O_2 *in vitro* in order to further understand the efficacy of this medicine.

The involvement of the Notch signaling pathway in the vaccarin-induced protective effects observed during H_2O_2 -induced cellular injury remains to be studied. In the present study, the role of the Notch signaling pathway in H_2O_2 -induced cellular injury in EA.hy926 cells was first explored. Furthermore, whether vaccarin protected EA.hy926 cells from H_2O_2 -induced cellular injury via Notch signaling pathway inhibition was investigated. To know whether vaccarin reduced Notch1 and apoptosis *in vitro*, the role of vaccarin on the reduction of apoptosis in EA.hy926 cells within the range of an effective concentration was evaluated.

Materials and methods

Drugs and chemicals. Vaccarin was purchased from Shanghai Shifeng Technology Co., Ltd. (Shanghai, China). Sulforhodamine B (SRB) was purchased from Sigma (St. Louis, MO, USA). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit was purchased from KeyGen (Nanjing, China). The 3,3'-diaminobenzidine (DAB) kit was purchased from Beyotime Institute of Biotechnology (Jiangmen, Jiangsu, China). The Notch1 (ab52627), Hes1 (ab108937), caspase-3 (ab32351) and β -tubulin (ab6046) antibodies were purchased from Abcam (Hong Kong, China). The goat anti-rabbit secondary antibody (AB10058) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The kits for the measurement of lactate dehydrogenase (LDH), methane dicarboxylic aldehyde (MDA), superoxide dismutase (SOD) and bicinchoninic acid (BCA) concentrations were purchased from the Institute of Jiancheng Bioengineering (Nanjing, Jiangsu, China). M-PER Mammalian Protein Extraction reagent was purchased from Thermo Scientific (Waltham, MA, USA).

Cell culture and treatments. Human EA.hy926 (ATCC CRL-2922) endothelial cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum (Gibco, Carlsbad, CA, USA) and incubated at 37°C in humidified air containing 5% CO_2 .

Prior to treatment with H_2O_2 , cells were grown to 80-90% confluence and placed in 2% serum-containing media for

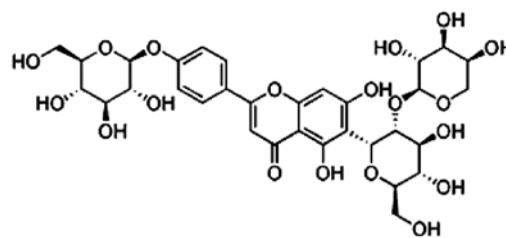


Figure 1. Chemical structure of vaccarin.

12 h to achieve cell synchronization. The vaccarin solution was diluted with culture medium immediately prior to the experiment. The cells were treated with H_2O_2 in the absence or presence of vaccarin. The cell monolayers were pretreated with vaccarin (3.44, 6.88 or 13.76 μ M) at 37°C for 24 h, and were subsequently induced by H_2O_2 . Following treatment with H_2O_2 , cells were maintained in 10% serum-containing media in a 5% CO_2 atmosphere at 37°C for further experiments.

Analysis of cell viability. The SRB assay was performed to assess EA.hy926 cell viability (35). EA.hy926 cells were seeded in 96-well culture plates (each concentration for four repeat holes) and cultured in medium for 24 h. Subsequently, three different final concentrations of vaccarin (3.44, 6.88 or 13.76 μ M, dissolved in serum-free medium) were added to each well. After 24 h incubation at 37°C, H_2O_2 solution (250, 500 or 1000 μ M) dissolved in serum-free medium was added to each well and cultivated (for 2 or 4 h). Subsequently, the medium was removed and 200 μ l 5% trichloroacetic acid (TCA) was added to each well to fix the cells for 40 min at 4°C. TCA solution was removed and replaced with 100 μ l SRB and incubated at 30°C for 30 min. Following this, SRB was removed and the cells were washed twice in deionized water. Finally 10% tris hydroxymethyl aminomethane (Tris) was used to dissolve the SRB and the samples were agitated for 30sec at room temperature twice. The results were determined at 540 nm using a Multiskan MK3 microplate reader (Thermo Labsystems, Milford, MA, USA) and the cell viability was expressed as an optical density (OD) value. In addition, the cell morphology was observed under an inverted/phase contrast microscope, and images were captured at 200 amplification with Olympus Nikon Eclipse Ti (Tokyo, Japan).

Cell migration assay. The migration rate was measured by the wound healing assay (36). Briefly, EA.hy926 cells (8×10^4 cells/well) were seeded and were cultured at 37°C in a saturated humidity containing 5% CO_2 for 24 h. When the cells have attached completely, the middle of the cell plate was scraped with a line ~1 mm in width following treatment of vaccarin (6.88 and 13.76 μ M). The cells were incubated and randomly chosen fields were photographed at 100 amplification under a microscope video system (Olympus, Nikon Eclipse Ti). The mean distance between the two ends of the scratch was quantified by manual measurements. The control was defined as 100%.

Measurement of LDH release and the intracellular contents of SOD and MDA. LDH, an indicator of cell injury, was detected with an assay kit according to the manufacturer's instructions. The activity of enzyme was expressed as units per liter, and the

absorbance was read at 450 nm. As described previously (37), the activities of SOD and MDA were determined using commercially available kits according to the manufacturer's instructions. The enzyme activities were expressed as units per milligram of protein. The assay for measuring SOD activity was based on the ability of SOD to inhibit the oxidation of hydroxylamine by O_2^{2-} produced from the xanthine-xanthine oxidase system. One unit of SOD activity was defined as the amount that reduced the absorbance at 450 nm by 50%. The experiment of BCA measurement was performed prior to determining of SOD. MDA was measured at 532 nm by its reaction with thiobarbituric acid to form a stable chromophoric product. The MDA level was expressed as nanomoles per milligram protein.

Cellular apoptosis assay. EA.hy926 cells were prepared for analysis according to the instructions of the Annexin V-FITC/PI Apoptosis Detection kit. The stained cells were quantitatively detected using the FACSscan flow cytometer (BD Biosciences, San Jose, CA, USA) in the FL1-H and FL2-H channels. Data were analyzed using Cell Quest Pro software (BD Biosciences). A total of 10,000 cells were analyzed.

Hoechst staining. In the 24-well plate with cover slips, after 6×10^4 EA.hy926 cells were seeded onto each well and cultured for 24 h, different doses of vaccarin (6.88 and $13.76 \mu\text{M}$) were applied and incubated 24 h before being subjected to 4 h of H_2O_2 (1000 μM) treatment. Following the removal of the culture medium, the cells were fixed with 0.5 ml 4% para-formaldehyde, and washed with phosphate-buffered saline (PBS) twice. After treatment with the Hoechst dyes (Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 10 min, the cells were rinsed with PBS twice. The stained cells were immediately observed under a fluorescence microscope (Olympus, Nikon Eclipse Ti).

Western blot analysis. Protein levels were analyzed by western blot as described previously (38). Briefly, 25 μg total protein/well was loaded after denaturing in loading buffer at 100°C for 5 min. The protein extracts were subjected to 8-12% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). Following the transfer, the membranes were blocked at room temperature for 2 h in 5% skimmed dry milk/TBST and were incubated at 4°C overnight with various primary antibodies. The primary antibodies are as follows: Notch1 (1:500 dilution), Hes1 (1:500 dilution), caspase-3 (1:1000 dilution) and β -tublin (1:1000 dilution). The following day, the membranes were washed three times with TBST for 10 min at room temperature, and were subsequently incubated in secondary antibody (anti-rabbit immunoglobulin G, 1:2000 dilution) conjugated to horseradish peroxidase for 2 h at room temperature. Following incubation, the membranes were washed as above, and the protein bands were visualized using the DAB-advanced western blotting detection kit. β -tublin was used as the protein loading control.

Statistical analysis. The results are presented as mean \pm standard deviation. Statistical analysis was performed by one-way analysis of variance test. $P < 0.05$ was considered to indicate a statistically significant difference.

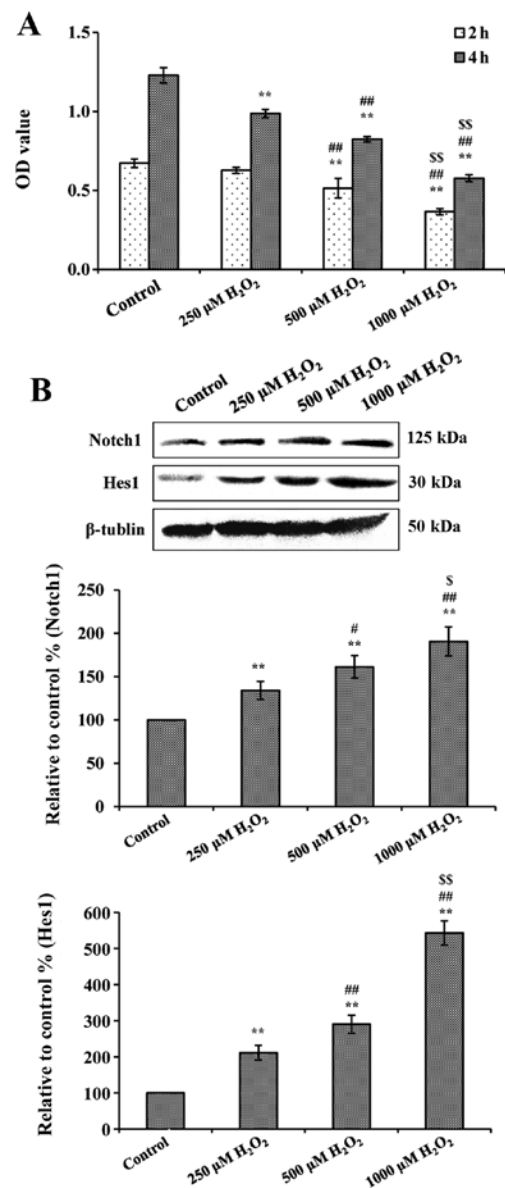


Figure 2. Effects of H_2O_2 on the viability of EA.hy926 cells and Notch1 and Hes1 expression. (A) The viability of EA.hy926 cells was assessed by performing an sulforhodamine B (SRB) assay, and is expressed as an optical density (OD) value. (B) Representative images of the western blots are shown (treatment for 24 h). The results are expressed as the means \pm standard deviation; $n=4$, $^{**}P < 0.01$ compared to the control group; $^{\#}P < 0.05$, $^{##}P < 0.01$ compared to the 250 μM H_2O_2 group; $^{\$}P < 0.05$, $^{ss}P < 0.01$ compared to the 500 μM H_2O_2 group.

Results

Effect of H_2O_2 on the viability of EA.hy926 cells and Notch1 and Hes1 expression. In order to investigate the effect on EA.hy926 cells induced by H_2O_2 , the cells were induced by H_2O_2 (250, 500 and 1000 μM) for 2 and 4 h and cell viability was examined with the SRB assay. As shown in Fig. 2A, treatment with H_2O_2 alone significantly reduced cell viability by $>50\%$ (after 4 h treatment at 1000 μM H_2O_2). The OD values in the control group were 0.673 ± 0.027 and 1.229 ± 0.049 after 2 and 4 h of culture. All the groups had a significant decrease compared to the normal groups, except 250 μM H_2O_2 after 2 h culture ($P < 0.01$, compared to respective control groups). Eventually, 4 h treatment with 1000 μM H_2O_2 was selected for the subsequent experiments.

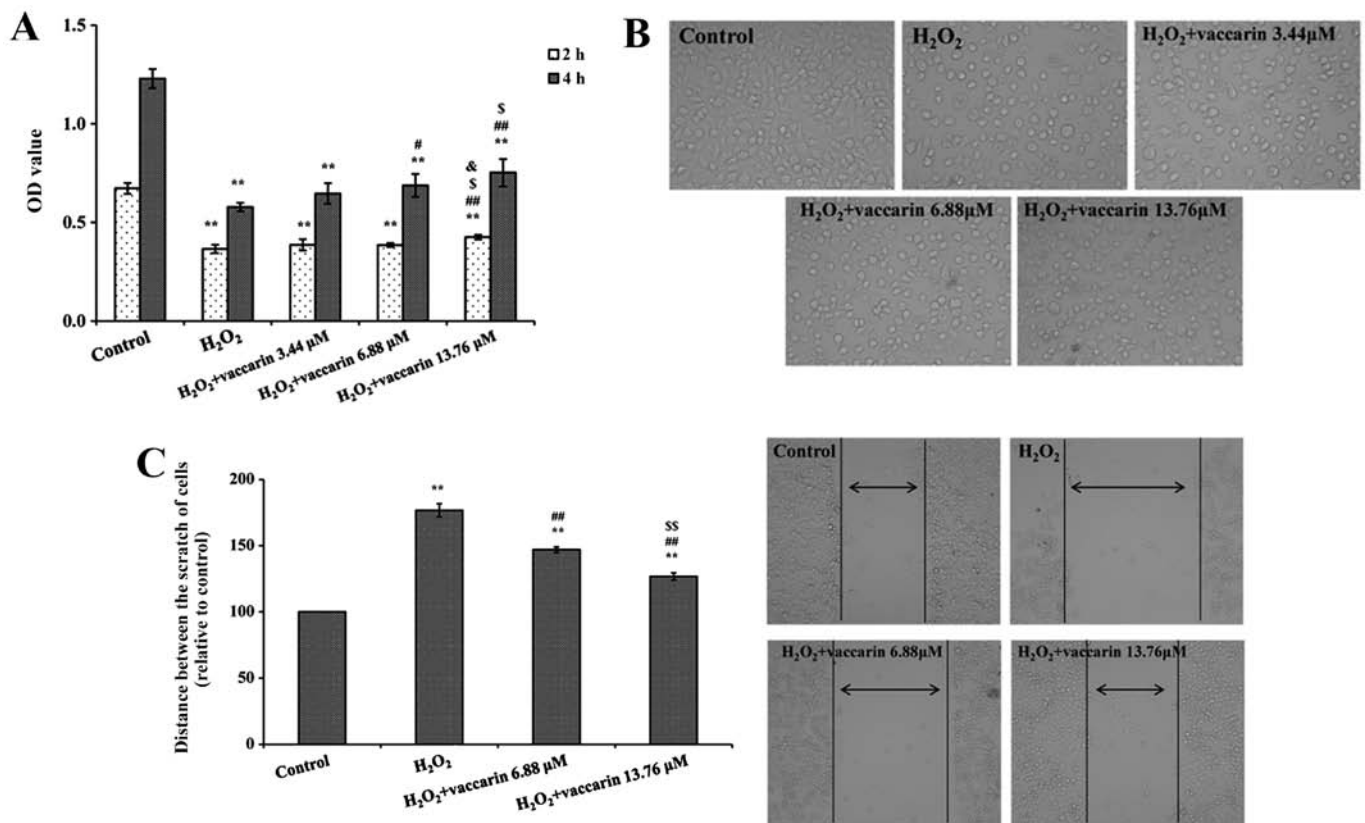


Figure 3. Effects of vaccarin on the viability, morphology and migratory ability of H₂O₂-injured EA.hy926 cells (treated for 4 h). (A) The viability of EA.hy926 cells was assessed by performing an sulforhodamine B (SRB) assay, and the viability is expressed as an optical density (OD) value. (B) The cell morphology (treated for 4 h) was observed under an inverted/phase contrast microscope, and images were captured. Significant cell shrinkage was observed in the H₂O₂ group. Additionally, vaccarin treatment reduced the H₂O₂-induced cell shrinkage. (C) The migratory ability of EA.hy926 cells was assessed by performing the wound healing assay, and the migratory ability is expressed as the mean distance between the two ends of the scratch (magnification, x100). The mean distance in the control group was set to 100%. The results are expressed as the means \pm standard deviation; n=4, *P<0.01 compared to the control group; [#]P<0.05, ^{##}P<0.01 compared to the 1000 μM H₂O₂ group; ^{\$}P<0.05, ^{\$}\$P<0.01 compared to the 1000 μM H₂O₂ + 3.44 μM vaccarin group; [&]P<0.05 compared to the 1000 μM H₂O₂ + 6.88 μM vaccarin group.

In addition, Notch1 and Hes1 expression were detected after 4 h H₂O₂ culture and the results indicated that H₂O₂ treatment significantly increased the expression of Notch1 and Hes1 in a dose-dependent manner (shown in Fig. 2B).

Effects of vaccarin on the viability and migratory ability of H₂O₂-injured EA.hy926 cells. The effects of vaccarin on EA.hy926 cell proliferation were examined after 2 and 4 h treatment with 1000 μM H₂O₂. As shown in Fig. 3A, the cell viability in the presence of vaccarin groups significantly increased compared to the absence groups of vaccarin (P<0.05 or P<0.01), respectively. Vaccarin provided dose-dependent protection against the reduction in cell viability induced by H₂O₂ for the concentration range, 3.44–13.76 μM. As observed under the microscope, H₂O₂ treatment resulted in significant cell shrinkage compared to the control group. However, pretreatment with three different vaccarin concentrations (3.44, 6.88 and 13.76 μM) attenuated H₂O₂-injured cell shrinkage (shown in Fig. 3B). Based on these results, pretreatment with 6.88 and 13.76 μM vaccarin and 1000 μM H₂O₂ for 4 h was chosen for further studies.

As shown in Fig. 3C, following treatment with H₂O₂ the migratory ability of cells was decreased and the distance between the scratch (μm) of cells was 176.68 \pm 4.89% (P<0.01,

compared to the normal cells). However, vaccarin (6.88 and 13.76 μM) treatment groups significantly decreased the distances (147.01 \pm 2.14% and 126.76 \pm 2.68%;, P<0.01, compared to the H₂O₂ group, respectively).

Effects of vaccarin on SOD, LDH release and MDA levels in H₂O₂-injured EA.hy926 cells. Treating the cells with H₂O₂ for 4 h decreased the SOD levels, but increased the LDH release and MDA levels (P<0.01, compared to the control group, respectively). As demonstrated in Fig. 4, following incubation of EA.hy926 cells in the presence of vaccarin (6.88 and 13.76 μM) with H₂O₂ significantly increased SOD activity (Fig. 4A) and decreased the level of MDA and LDH release, respectively (Fig. 4B and C). According to these results, vaccarin significantly changed the SOD activity, LDH leakage and MDA level in H₂O₂-induced endothelial cells in a concentration-dependent manner.

Effects of vaccarin on the apoptotic index of H₂O₂-injured EA.hy926 cells. Induction of apoptosis was measured by Annexin V and PI-double staining. The flow cytometric analysis of the H₂O₂ group showed an increase in apoptosis. The ratio of prophase and late apoptosis reached 78.22 \pm 0.83% and 2.00 \pm 0.22%, respectively (P<0.01, compared to the control

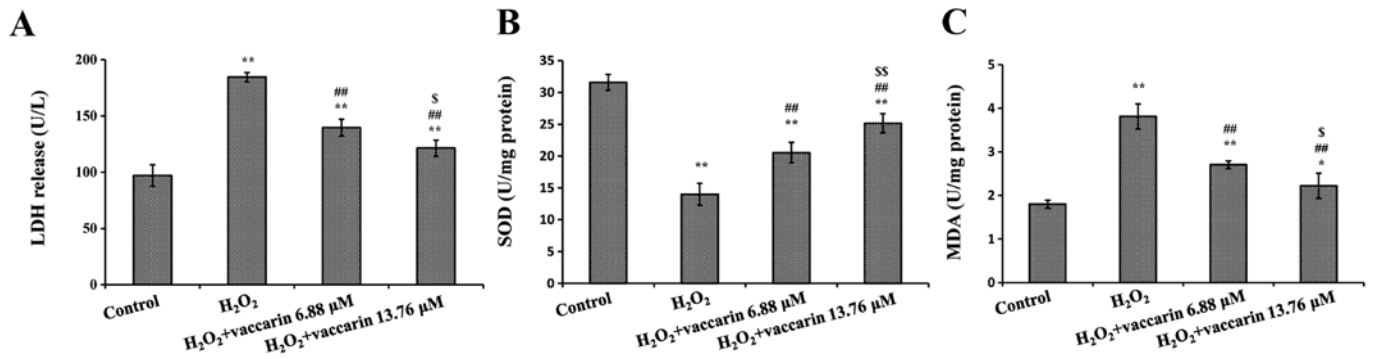


Figure 4. Effects of vaccarin on the intracellular superoxide dismutase (SOD), malondialdehyde (MDA) levels and lactate dehydrogenase (LDH) release by H₂O₂-injured EA.hy926 cells (treated for 4 h). (A) The release of LDH in the EA.hy926 cells treated with H₂O₂ in the absence or presence of vaccarin. (B) The intracellular SOD level in the EA.hy926 cells treated with H₂O₂ in the absence or presence of vaccarin. (C) The intracellular MDA level of the EA.hy926 cells treated with H₂O₂ in the absence or presence of vaccarin. The results are expressed as the means ± standard deviation; n=4, *P<0.05, **P<0.01 compared to the control group; ##P<0.01 compared to the 1000 μM H₂O₂ group; ^SP<0.05, ^{SS}P<0.01 compared to the 1000 μM H₂O₂ + 6.88 μM vaccarin group.

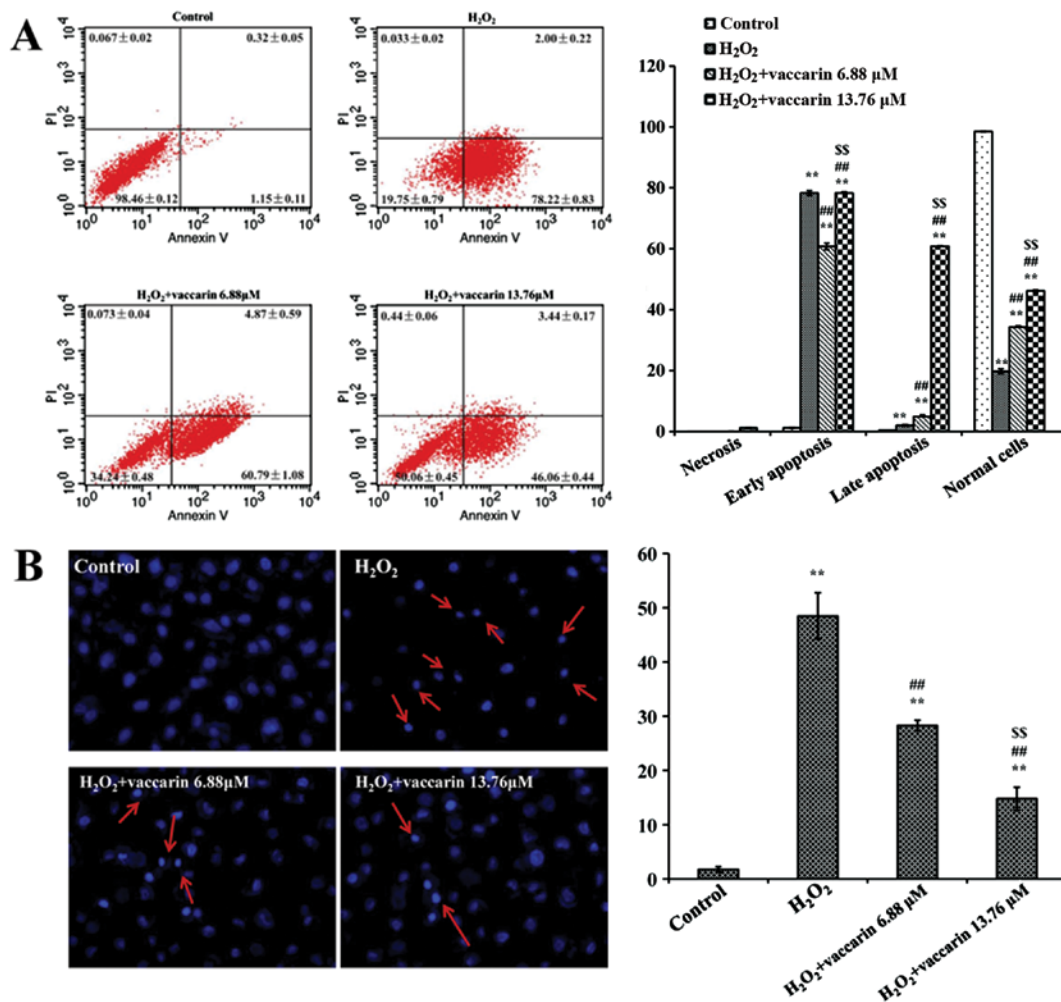


Figure 5. Effects of vaccarin on cell apoptotic rate of H₂O₂-injured EA.hy926 cells (treated for 4 h). (A) The apoptosis of EA.hy926 cells was assessed by performing a Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide Apoptosis Detection kit, and cellular apoptosis is expressed as the apoptotic index. (B) Hoechst 33342 staining was performed to stain the nuclei of the cells (blue); the morphology of the normal cell nucleus was different from the injured cells. The apoptotic index is expressed as: (number of positively stained apoptotic cells/total number of cells counted) × 100%. The results are expressed as the means ± standard deviation; n=4, **P<0.01 compared to the control group; ##P<0.01 compared to the 1000 μM H₂O₂ group; ^{SS}P<0.01 compared to the 1000 μM H₂O₂ + 6.88 μM vaccarin group.

group). However, the apoptosis ratio for the treatment with vaccarin (6.88 and 13.76 μM) groups significantly reduced (P<0.01, compared to the H₂O₂ group, shown in Fig. 5A).

In the Hoechst stain experiment, it was also found that apoptosis was significantly higher in the H₂O₂ group (shown in Fig. 5B). Following treatment with vaccarin (6.88 and

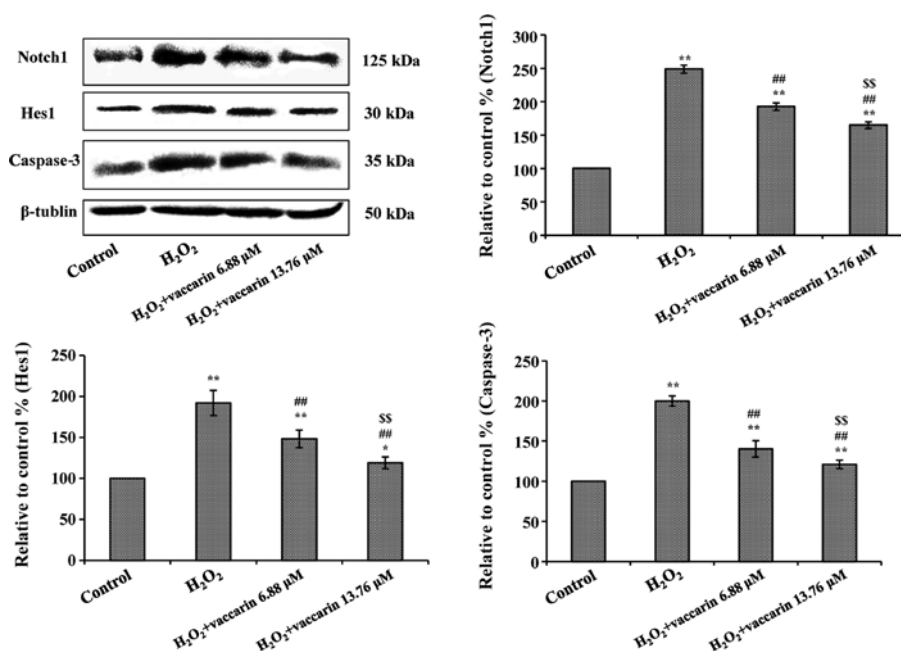


Figure 6. Effects of vaccarin on the expression of Notch1, Hes1 and caspase-3 in the H_2O_2 -injured EA.hy926 cells (treated for 4 h). Representative images of the western blots are shown. The results are expressed as the means \pm standard deviation; n=4, *P<0.05, **P<0.01 compared to the control group; ##P<0.01 compared to the 1000 μM H_2O_2 group; ^{SS}P<0.01 compared to the 1000 μM H_2O_2 + 6.88 μM vaccarin group.

13.76 μM), the cell index was markedly decreased ($P<0.01$, compared to the H_2O_2 group).

Effects of vaccarin on the expression of Notch1, Hes1 and caspase-3 in H_2O_2 -injured EA.hy926 cells. To further investigate the effect and mechanism of vaccarin in H_2O_2 -injured EA.hy926 cells, the expression of Notch1, Hes1 and caspase-3 were detected through western blotting analysis. As demonstrated in Fig. 6, treatment with H_2O_2 significantly increased the expression of Notch1, Hes1 and caspase-3 (% relative to control group, $P<0.01$). When the cells were pretreated with vaccarin (6.88 and 13.76 μM), the expression of Notch1, Hes1 and caspase-3 decreased significantly ($P<0.01$, compared to the H_2O_2 group).

Discussion

Endothelial barrier dysfunction plays a critical role in the pathogenesis of diabetic vascular complications (39). Exposure of the vascular endothelial tissue to H_2O_2 causes endothelial dysfunction and further complications of atherosclerosis and hypertension (40). A previous study showed that H_2O_2 induced reactive oxygen species (ROS), which can cause cellular dysfunction, cell apoptosis and cell death (41). Notch signaling has been widely connected in endothelial to mesenchymal transformation (42), endothelial cell proliferation (43) and apoptosis control (44). In the cultured renal proximal tubular cell model, puromycin aminonucleoside induced the upregulation of the Notch1 signaling components, such as Notch intracellular domain and the downstream molecule Hes1, and also the downregulation of Numb, an intrinsic Notch antagonist (45).

The present study has indicated that in human EA.hy926 endothelial cells, H_2O_2 caused vascular endothelial cell apoptosis via activating Notch1 and Hes1. Vaccarin contributed

a protective effect against H_2O_2 induced endothelial injury, as shown by improved cell viability, migratory ability and a decreased apoptotic index. The protective effects of vaccarin against cell injury are, in part, dependent on Notch1 inhibition. Numerous anti- H_2O_2 -induced cell injury drugs protect against apoptosis by regulation of the cell apoptosis pathway (46-47). B-cell lymphoma 2, Bax, Bak and caspase-3 have significant roles in cell apoptosis and are important members of the cell survival pathway (48-49). Caspase-3 is the main component of the execution steps of the apoptotic process, and the activation of caspase-3 is the central link of apoptosis (50). A previous study showed that caspase-3 may be a main target involved in the ROS-mediated H_2O_2 -induced apoptosis in human endothelial cells (51). In the present study, vaccarin was shown to effectively suppress caspase-3 overexpression induced by H_2O_2 .

H_2O_2 -induced free radicals can have irreversible effects on a number of biomolecules, including lipids, leading to lipid peroxidation. LDH leakage, which is associated with membrane damage, and MDA, a by-product of lipid peroxidation induced by excessive ROS exposure, are commonly used biomarkers of oxidative stress injury (37). Antioxidants, such as SOD, are important in providing protection against H_2O_2 injury. Thus, the combined action of SOD and other endogenous antioxidants can reduce intracellular ROS (37). In the present study, significant decreases in SOD were observed in EA.hy926 cells following H_2O_2 exposure, indicating the impairment in antioxidant defenses. In addition, a clear increase in MDA production was correlated with an increase of LDH release. Preincubation with vaccarin protected EA.hy926 cells from H_2O_2 -induced cellular oxidative injury as shown by inhibition of the levels of LDH and MDA, but increased SOD activity. Notably, in addition to downregulation of H_2O_2 -induced Notch signaling, vaccarin treatment also downregulated H_2O_2 -induced the

apoptotic pathway-related protein, caspase-3. These results indicate that increased endogenous antioxidant preservation and attenuation of the cell apoptotic pathway may represent a major mechanism of cellular protection by vaccarin.

In conclusion, the present study demonstrates that vaccarin, the major bioactive compound of *Vaccariae semen*, can prevent H₂O₂-induced apoptosis of human EA.hy926 endothelial cells. Vaccarin was effective on attenuating cells injury via the inhibition of the Notch signaling pathway. These findings indicated that vaccarin has anti-apoptotic activity in the diabetic vascular lesion, which leads to the development of atherosclerosis or hypertension. The study shows that vaccarin is a possible therapeutic in the prevention of diabetic vascular lesion or atherosclerosis.

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