

Influence of Several Ophiopogon Japonicus Extracts on Expression of ICAM-1, VEGF, Bcl-2 in Damaged HUVEC Induced by Hydrogen Peroxide*

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ABSTRACT Objective: To investigate the influence of different Ophiopogon Japonicus extracts on expression of intercellular adhesion molecule -1 (ICAM-1) and VEGF, Bcl-2 in damaged human umbilical venous endothelial cell (HUVEC) induced by hydrogen peroxide. **Methods:** HUVECs were cultured in vitro, and HUVEC damage model was induced by using hydrogen peroxide (H₂O₂). Blue methyl thiazolyl tetrazolium (MTT) method was used to detect the number of survival cells, the expression of ICAM-1 on the surface of HUVEC was analyzed by flow cytometry and immunocytochemical method was used to detect the distribution of VEGF, Bcl-2 in HUVEC. **Results:** Compared with the normal group, the cell proliferate activity of model group was significantly decreased ($P < 0.01$), while after being treated by water extract and butanol extract from Ophiopogon Japonicus, it was significantly increased compared with the model group ($P < 0.05$, $P < 0.01$). The measurement of flow cytometry showed that the n-butanol extract could reduce the increased gene expression of ICAM-1 caused by hydrogen peroxide. For Bcl-2 expression, the model group was much less than the normal control group, and Ophiopogon Japonicus extract group was significantly higher than the model group ($P < 0.01$). For VEGF expression, the model group was much higher than the normal group, and aqueous extract and n-butanol extract of Ophiopogon Japonicus group was higher than the model group ($P < 0.05$, $P < 0.01$). **Conclusion:** Ophiopogon Japonicus extract, especially the extract by n-Butanol could be used for anti-apoptotic, promoting proliferation, reducing expression of ICAM-1.

Key words: Ophiopogon Japonicus; Human umbilical venous endothelial cell (HUVEC); VEGF; Hydrogen peroxide(H₂O₂); ICAM-1; Bcl-2

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Introduction

Traditional Chinese medicine believes in that Ophiopogon Japonicus has the efficacy of clearing heart heat and nourishing Lung Yin. Our previous researches had proved the antioxidative effect of Ophiopogon Japonicus and it could significantly protect HUVEC by removing free radicals. Ophiopogon Japonicus is an important drug in traditional Chinese medicine to treat cardio-cerebrovascular disease^[1-2]. The effective parts and effective compounds are the basis of therapeutic effects in traditional Chinese medicine. They can be used to reduce drug toxicity and improve therapeutic effects. This is an important way for exploring the mechanism of traditional Chinese medicine. This project is designed to study the effective compounds of Ophiopogon Japonicus and its mechanisms of preventing cardio-cerebrovascular disease by using the damaged HUVEC model which induced by hydrogen peroxide (H₂O₂) and observe the influence of n-butanol and water extracts of different parts of Ophiopogon Japonicus on the expression of ICAM-1, VEGF, Bcl-2.

1 Materials and Methods

1.1 Materials

Ophiopogon Japonicus (Thumb.) Ker-Gawl, was purchased

from Mianyang, Sichuan Province, and identified by the department of authentication of Chinese medicine, in NJUTCM as the certified products.

1.2 Extraction and Separation

Dried root tuber of Ophiopogon Japonicus (10 kg), was dried for 8 hours under 60 °C, smashed into powder, sifted through a 180-mesh-sieve and evenly mixed, then was soaked in 100 L of 70% ethanol for 12 hours. After those above, filtrated the solvent after a hot-reflux-extraction for 2 hours, repeated the extraction twice, then mixed the extracts, and recovered the solvent until there was no odor of alcohol. Kept on extracting the solvent by using water-saturated n-butanol, till got the water extract and the water-saturated n-butanol extract. Finally, recovered the solvent separately and got the extract by vacuum drying.

1.3 Preparation for materials

The water extract and n-butanol extract was dissolved in Dimethyl Sulfoxide (DMSO) (concentration of DMSO <0.01%). Then 0.01% DMSO was used as the solvent control group to test the toxicity to cells. The result showed that the DMSO solution had no cytotoxicity to the cells. Finally, the solvent was prepared to be the clinical recommended dosage of human (9 g) for stock by using the extracted parts that are dissolved in serum-free culture medium (RPMI). The dissolved extracted parts were mixed with

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serum-free RPMI-1640 culture medium and the concentration was equivalent to clinical dosage for human use (9 grams). The solution was reserved for experiments.

1.4 Instruments and reagents

1. RPMI-1640 culture medium (GIBCO, USA) (Ph7.4, contain 10% FBS, L-glutamine 0.33 g, penicillin 100 U and streptomycin 100 U per liter), 2. Trypsin (MERCK, USA), 3. H_2O_2 (Analytical reagent, Zhenjiang chemical reagent factory, batch number: 2001111), 4. Dimethyl Sulfoxide (DMSO) (MERCK, GERMANY), 5. 6-well and 96-well culture plate (Costar, Denmark), 6. Incubator (NAPCO, USA), 7. Inverted optical microscope (OLYMPUS, Japan), 8. Absolute alcohol (Analytical reagent, Zhenxing chemical reagent factory Shanghai, batch number: 20021200365), 9. PI (Sigma, USA), 10. Flow cytometry (BD, USA), 11. SABC immunohistochemistry kit and DAB colour-showing kit (Boster, Wuhan).

1.5 Methods

1.5.1 Culturing endothelial cell in vitro

Human umbilical venous endothelial cells (HUVEC), were purchased from Oceanic Pharmaceutical Institute of NJUTCM and cultured in RPMI-1640 culture medium (containing 10% FBS).

1.5.2 Grouping

The Well-grown-HUVECs were digested by 0.25% trypsin, blown lightly, and the cell suspension was obtained. Then the cell concentration was adjusted to 5×10^6 cells/ml and the HUVEC was incubated to the 6-well culture plate. When the bottom of the culture plates were covered with cells (about 24 hours later), the supernatant was removed, and replaced by serum-free medium for 24 hours. Finally, the HUVECs were divided into 4 groups: 1. Normal control group (no inducers), 2. Model group (containing serum-free medium with $100 \mu\text{mol} \cdot \text{L}^{-1} H_2O_2$), 3. Water extract-treated group (H_2O_2 and water extract of *Ophiopogon Japonicus*), 4. N-butanol extract group (H_2O_2 and n-butanol extract of *Ophiopogon Japonicus*). The final concentration of each composition is $12.9 \text{ mg} \cdot \text{L}^{-1}$.

1.5.3 Measuring the viability of cells by blue methyl thiazolyl tetrazolium (MTT)

The 3rd generation of HUVEC was digested by 0.25% trypsin and blown lightly to obtain the single-cell-suspension. Then the cells were adjusted to a concentration 5×10^6 cells/ml and inoculated to 96-well culture plates. In each culture plate, the 1st and 2nd rows contained serum without cells in order to eliminate the influence of serum composition on absorbance value (AV), the 3rd and 4th rows contained the cell suspension of HUVEC, the 5th and 6th rows contained the cell suspension of HUVEC with H_2O_2 , the 7th and 8th rows contained the cell suspension with water extract, the 9th and 10th rows contained the cell suspension with n-butanol extract. All the culture plates were fixed

in an incubator with 5% CO_2 at 37°C , and each well of the plates was added with $20 \mu\text{l}$ of MTT 24 hours after administration of the composition. After another 4 hours incubation, the supernatant was carefully removed and $150 \mu\text{l}$ of DMSO was added in order to dissolve the crystals, then the absorbance value of each well was measured by enzyme-labeled meter 490nm after 10-minutes oscillation. The cell growth inhibition rate is: (calculation formula) $IR(\%) = \frac{(\text{the OD value of control group} - \text{the OD value of detection group})}{\text{the OD value of control group}} \times 100\%$.

1.5.4 Detecting the gene expression of ICAM-1 by flow cytometry

5 ml cell culture with the cell concentration of 1×10^6 cells/ml was inoculated for 24 hours and synchronized for 24 hours. After that, the cells were treated with corresponding composition and collected 24 hours after the administration. The cell layer was washed with phosphate buffer saline (PBS) for 3 times, then the supernatant was removed. Cells were re-suspended and $100 \mu\text{l}$ of the solution was taken and $20 \mu\text{l}$ of ICAM-1 was added to each tube, and then incubated without light for 30 minutes. After all those steps, the solution would be washed continuously with PBS for 2 times, removed the supernatant, and collected suspended cells after adding $300 \mu\text{l}$ of PBS. Finally, the median fluorescence intensity (MFI) of cells would be measured by flow cytometry, and the gene expression of ICAM-1 would be analyzed by analysis software Cell Quest.

1.5.5 Detecting VEGF, Bcl-2 by immunohistochemistry

The HUVECs were inoculated to the pre-treated cover glass and cultured separately after the adhering to the wall. The HUVECs would be added H_2O_2 extracts of different groups and cultured for 24 hours, fixed with polymerisatum for 30 minutes, washed with PBS (pH7.4), and incubated for 20 minutes with normal goat serum. The primary antibodies were fixed under the temperature of 4°C overnight and washed by PBS. The secondary antibodies were incubated under normal room temperature for 20 minutes and washed by PBS. By using the fresh-compounded DAB solution as the reagent, the positive expression of Bcl-2 shown in cytoplasm was tan granules, while the positive expression of VEGF primary shown in cytoplasm was purplish red granules. Finally, analyzed the immunohistochemical images result by image analyzer, and the expression of positive signals would be shown through the mean OD values measured earlier.

1.6 Statistics analysis

The sample size of each group was $n=6$. All the results were shown as $\bar{x} \pm s$. The experimental data analyses were conducted by the statistics software (SPSS 12.0), and the one-way ANOVA was used for the comparison among groups.

2 Results

2.1 Measurements by blue methyl thiazolyl tetrazolium (MTT)

According to table 1, H₂O₂ had significant inhibitory effects on HUVEC, the OD value decreased significantly compared with the control group (P<0.01). Both the water extract and n-butanol

extract of Ophiopogon Japonicus could inhibit decrease of OD value caused by H₂O₂, compared with the model group (P<0.05, P<0.01 respectively).

Table 1 Influence of the different extracts from radix of Ophiopogon Japonicus on H₂O₂ damaging the activity of human umbilical vascular endothelial cell (HUVEC)

Groups	MTT OD ($\bar{X} \pm S$)	IR(%)
Control	0.5118 $\pm$ 0.0484*	
Model	0.2833 $\pm$ 0.0363*	44.64
Water extract	0.3468 $\pm$ 0.0732 Δ *	32.24
n-butanol extract	0.4226 $\pm$ 0.0585*	17.43

Note : *P<0.01 compared with normal group*P<0.01; compared with model group Δ P<0.05, *P<0.01

2.2 Detecting the gene expression of ICAM-1 by flow cytometry

The result suggested the expression of ICAM-1 by HUVECs increased from 0.37% to 1.58% (normal group) after treated with H₂O₂, while the expression of ICAM-1 by HUVEC decreased to 1.32% after treated with n-butanol extract of Ophiopogon Japonicus.

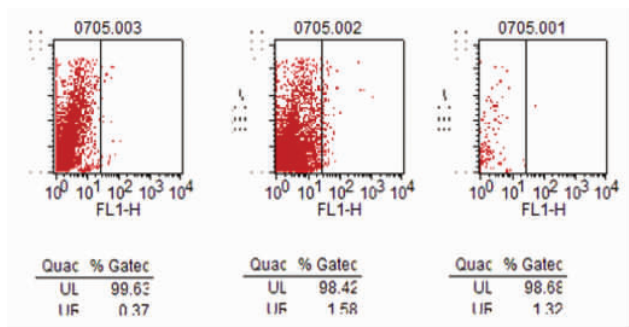


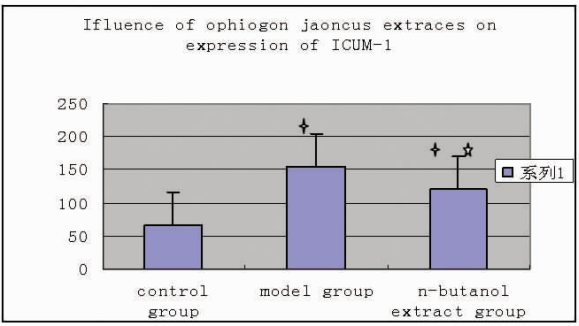
Fig.1 Effect of n-butanol fraction extracts of Ophiopogon Japonicus on expression of ICAM-1 in H₂O₂ damaging HUVEC detected by flow cytometry

The value was measured by flow cytometry and U test of Poisson distribution analysis. The expression of ICAM-1 of HUVECs from model groups was significantly increased (compared with the normal control group u=13.57 P<0.05), indicating that H₂O₂ could promote the expression of ICAM-1. The expression of ICAM-1 of n-butanol group was increased when compared with the normal group (u=8.52 P<0.05); While compared with the model group the expression of ICAM-1 of HUVEC was decreased (u=6.054 P<0.05).

Table 2 Average optical density value comparison by the influence of the different extracts from radix of Ophiopogon Japonicus on H₂O₂ injured human umbilical vascular endothelial cell (HUVEC) VEGF and Bcl-2

Groups	VEGF OD ($\bar{X} \pm S$)	Bcl-2 OD ($\bar{X} \pm S$)
Control	0.1480 $\pm$ 0.0114*	0.3417 $\pm$ 0.05707 Δ
Model	0.2160 $\pm$ 0.0415*	0.2750 $\pm$ 0.01049 Δ
Water extract	0.2303 $\pm$ 0.0381 Δ *	
n-butanol extract	0.2471 $\pm$ 0.0589**	0.3283 $\pm$ 0.05115 Δ

Note: compared with normal group Δ P<0.05,*P<0.01; compared with model group Δ P<0.05, *P<0.01.

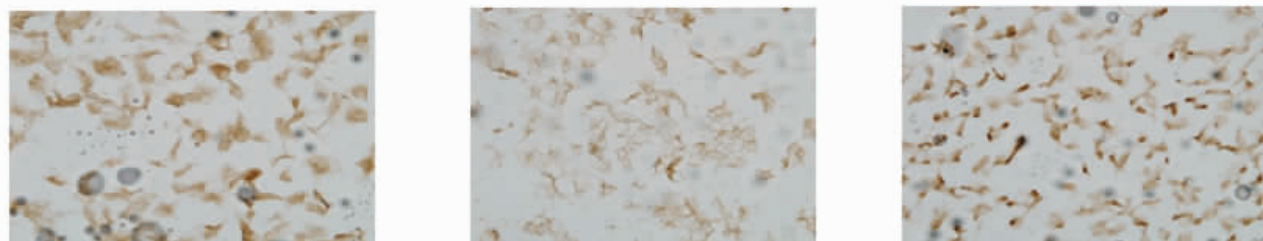


Note: compared with normal group P<0.05; compared with model group P<0.05

Fig.2 Effect of n-butanol extracts of Ophiopogon Japonicus on expression of ICAM-1 in H₂O₂ damaging HUVEC detected by flow cytometry

2.3 Detecting VEGF, Bcl-2 by immunohistochemistry

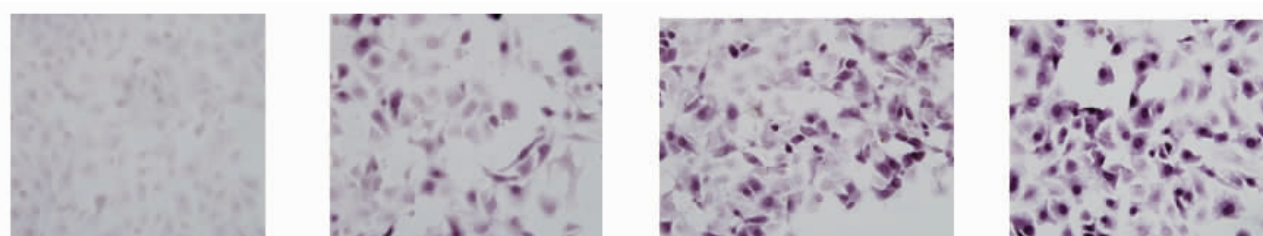
Mean OD value measured by the image analytic system showed that the expression of VEGF of HUVEC from other groups significantly increased (compared with the normal control group P<0.01) and H₂O₂ could promote the expression of VEGF of HUVEC. The result of Ophiopogon Japonicus extract group was similar to that of model group. The expression of VEGF in Ophiopogon Japonicus extract group and n-butanol group increased when compared with model group (P<0.05, P<0.01) respectively. For Bcl-2, compared with control group, the expression of model group significantly decreased (P<0.05), while its expression in n-butanol group significantly increased than that of the model group (P<0.05) (Table 2, Figure 2 and Figure 3).



1. control group× 200

2. model group× 200

3. n-butanol extract group× 200

Fig. 3 Effect of n-butanol fraction extracts of *Ophiopogon Japonicus* on expression of Bcl-2 in HUVEC detected by cell immunohistochemistry

1. control group× 200

2. model group× 200

3. Water extract group× 200

4. n-butanol extract group× 200

Fig.4 Effect of *Ophiopogon Japonicus* extracts on expression of VEGF in HUVEC detected by cell immunohistochemistry

3 Discussion

The damage of endothelial cells plays a very important role in the development of cardiovascular disease, while one of its inducing factorS is active oxygen. The damage to cells by H_2O_2 is always the research focus in this field. The transient increase of H_2O_2 could happen under several pathological conditions, for example, activation of polymorph nuclear leucocytes in vivo could produce $200 \mu\text{mol} \cdot \text{L}^{-1}$ of H_2O_2 , yet smoking could also induce $50 \sim 100 \mu\text{mol} \cdot \text{L}^{-1}$ of H_2O_2 . It is proved that H_2O_2 is relevant to the development of cardiovascular disease, such as coronary heart disease, hypertension and atherosclerosis [3]. It is reported that H_2O_2 could induce cell apoptosis: when the endothelial cells were exposed to low concentration of H_2O_2 , they would not show necrosis and dissolution but apoptosis, which is a concentration-and-time dependent manner [4]. But high concentration of H_2O_2 could cause the necrosis and dissolution of cells. Our previous studies were in consistence with the studies conducted by other researchers: $100 \mu\text{mol} \cdot \text{L}^{-1}$ of H_2O_2 could significantly increase the apoptosis rate of cells [5]. Apoptosis has been considered as the pathological basis of the pathogenesis and development of many kinds of diseases, such as trauma, shock, infection and cardiovascular disease [1]. Bcl-2 is the most representative inhibitory factor of apoptosis. It could delay or inhibit cell apoptosis caused by many factors, for example, some scholars had noticed that overexpression of Bcl-2 gene could protect the apoptosis of endothelial cells caused by inflammatory mediators or chemical factors [6]. Our experiment showed the expression of Bcl-2 of H_2O_2 group decreased, while the expression of Bcl-2 increased after *Ophiopogon Japonicus* extracts being ap-

plied, this suggested that *Ophiopogon Japonicus* extracts could protect HUVEC by decreasing the apoptosis of endothelial cells caused by H_2O_2 .

The integrity of vascular endothelium is not only depending on the decreasing of cell death, but also on keeping homeostasis between proliferation and apoptosis of vascular endothelial cells. As a result, effectively improving the proliferation and decreasing the apoptosis of endothelial cells could benefit the repair of injured endothelial cells. In recent years, VEGF is always under the spotlight because of its effects on regenerating the endothelium and blood vessels, and there are many reports about applying exogenous VEGF to vascular endothelial cells. Some investigations even noticed that VEGF could function against the apoptosis of endothelial cells caused by H_2O_2 [5]. VEGF is a special mitogen of vascular endothelial cells, it could promote the growth of vascular endothelial cells in vitro while induce angiogenesis in vivo. VEGF plays an important role in protecting the arteries during the course of disease by keeping the integrity of injured vascular endothelium as an endogenous regulator. It could promote the division and proliferation of endothelial cells efficiently and benefit the repair of injured vascular endothelium rapidly. According to some studies, the expression level of VEGF in normal cells is very low, yet the damage caused by H_2O_2 could increase the expression and low concentration of H_2O_2 could promote the proliferation of vascular endothelial cells significantly [4,7]. However, the normal physiological function of VEGF is impaired when applying the exogenous H_2O_2 and VEGF simultaneously, instead of be coordinated in promoting the proliferation of both H_2O_2 and VEGF [4]. In our experi-

ment, VEGF expressed in a low level in normal control group, while the expression of VEGF in model group increased, but the proliferative activity of cells reduced significantly. Compared with the model group, the VEGF expression of *Ophiopogon Japonicus* extracts group increased significantly and the proliferative activity of cells also increased. This implies *Ophiopogon Japonicus* extracts may have the functions of removing free radicals and anti-lipid peroxidation, both of which could reduce the injury caused by H_2O_2 and promote the VEGF expression of cells, so that increase the proliferative activity of cells and accelerate the vascular endothelium's renovation.

William et al. found that H_2O_2 could induce and increase the expression of ICAM-1 [8]. By activating the expression of ICAM to increase the adhesion to endothelium for white cells, H_2O_2 could aggravate the injury of vascular endothelium [9-10]. ICAM-1 is a kind of single-strand glycoprotein on the surface of cells, whose gene is located in the chromosome 19. ICAM-1 is an important adhesion molecule distributed over several kinds of cells, of which the highest expression was observed on the vascular endothelial cells. The result of our study showed that the expression of ICAM-1 in normal endothelial cells was weak, but the expression would be significant enhanced after being applied with H_2O_2 to activate HUVEC, while n-butanol extract from *Ophiopogon Japonicus* could reduce it. Our previous studies showed that H_2O_2 could significantly increased the expression of P65, which is the gene product of NF- κ B, however, this expression would be reduced after we applied n-butanol extract of *Ophiopogon Japonicus* [10]. According to these studies, we hypothesize that the reduction of the expression of ICAM-1 might be relevant to the following mechanism, 1. HUVEC was damaged by H_2O_2 ; this triggered the intracellular signal transduction mechanism and increased the activation of Ik Ba phosphokinase. 2. Ik Ba would be dissociated with NF- κ B after being phosphorylated, then NF- κ B quickly started the procedure of nuclear transfer and bound to the promoter or enhancer of target gene, which induced the synthesis of mRNA of the target gene. 3. The expression of ICAM-1 would be increased due to the down-stream regulation under control of NF- κ B. However, if drugs restored NF- κ B to the non-activation state, and NF- κ B transferred from nucleus to cytoplasm, the expression would be decreased. Those mentioned above showed n-butanol extract from *Ophiopogon Japonicus* could inhibit the expression of adhesion molecule on the surface of endothelial cells activated by H_2O_2 . ICAM-1 is the linkage between white cells and endothelial cells. Adhesion molecule plays an important role in this process. Blocking the expression of ICAM-1 might influence the adhesion between white cells and endothelial cells, which is an important part of the pathological changes of inflammation and atherosclerosis.

This makes a significant contribution to the protection of cardio-cerebrovascular system.

This study showed the extracts from different parts of *Ophiopogon Japonicus* could protect the HUVEC from damage caused by H_2O_2 in different degrees. The result of the cell activity measurement showed that two extract parts from *Ophiopogon Japonicus* could protect the vascular endothelial cells from damage caused by H_2O_2 , both n-butanol extract and water extract could increase the expression of VEGF and reduce the expression of ICAM-1 significantly. It suggested *Ophiopogon Japonicus* extracts, especially the extract by n-butanol, could be used for anti-apoptotic, promoting proliferation and reducing expression of ICAM-1. The active ingredients for protecting cardio-cerebrovascular system of *Ophiopogon Japonicus* concentrated in the part extracted by n-butanol^[11]. This result provided the new evidences for preventing and treating cardio-cerebrovascular disease by *Ophiopogon Japonicus*, but more researches are needed to explain whether this protection could be applied to endothelial cells in the whole body.

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麦冬不同提取物对过氧化氢损伤人血管内皮细胞 ICAM-1、VEGF、Bcl-2 表达的影响 *

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摘要 目的 观察麦冬不同提取物对过氧化氢诱导的人脐静脉内皮细胞(HUVEC)间黏附分子-1(ICAM-1)和 VEGF、Bcl-2 表达的影响。方法 体外培养 HUVEC,用过氧化氢(H₂O₂)制造 HUVEC 损伤模型。以四甲基偶氮唑蓝(MTT)比色法检测细胞存活数量,用流式细胞仪检测 HUVEC 表面 ICAM-1 的表达量;免疫细胞化学方法检测 HUVEC 的 VEGF、Bcl-2 的分布情况。结果 模型组较正常对照组细胞增殖活性明显降低(P<0.01)。与模型组相比,经麦冬水提物、正丁醇提取物处理组细胞增殖活性明显增加(P<0.05, P<0.01)。流式细胞仪检测显示正丁醇提取物可降低过氧化氢增加的 ICAM-1 基因的表达。Bcl-2 的表达,模型组明显低于正常对照组,而正丁醇组表达明显高于模型组(P<0.01)。VEGF 的表达,模型组明显高于正常对照组,麦冬水提物、正丁醇提取物处理组高于模型组(P<0.05 P<0.01)。结论 麦冬提取物具有抗凋亡、促增殖、降低细胞间黏附分子-1 表达的作用,尤以正丁醇提取物效果更为显著。

关键词 麦冬;血管内皮细胞;VEGF;过氧化氢;ICAM-1;Bcl-2

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