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Original Article

# Protective Effects of Anthocyanins Extracted from *Vaccinium Uliginosum* on 661W Cells Against Microwave-Induced Retinal Damage

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**ABSTRACT** **Objective:** To study the protective effect of anthocyanins extracted from *Vaccinium Uliginosum* (VU) on retinal 661W cells against microwave radiation induced retinal injury. **Methods:** 661W cells were divided into 6 groups, including control, model [661W cells radiated by microwave (30 mW/cm$^2$, 1 h)] and VU groups [661W cells pretreated with anthocyanins extracted from VU (25, 50, 100 and 200 μg/mL, respectively) for 48 h, and radiated by microwave 30 mW/cm$^2$, 1 h]. After treatment with different interventions, the cell apoptosis index (AI) was determined using Heochst staining; contents of malonaldehyde (MDA), glutataione (GSH), and activity of superoxide dismutase (SOD) were measured. mRNA expressions of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase 1(HO-1) were detected by real time quantitative polymerase chain reaction, and the expression of HO-1 protein was examined by Western blot analysis. Nucleus and cytoplasm were separated and Nrf2 protein expression was further verified by Western blot analysis. **Results:** There was significant difference in AI among the groups ($F$=322.83, $P$<0.05). Compared with the control group, AI was significantly higher in the model group and was lower in 4 VU-pretreated groups ($P$<0.05). Linear regression analysis showed the decline of AI was in a dose-dependent manner with VU treatment ($r$=0.8419, $P$<0.05). The MDA and GSH contents of 661W cells in VU-treated groups were significantly lower than the model group ($P$<0.05). Compared with the model group, the SOD activity in the VU-treated groups (50, 100 and 200 μg/mL) was significantly higher (all $P$<0.05). The Nrf2 and HO-1 mRNA expressions were slightly increased after irradiation, and obviously increased in 100 μg/mL VU-treated group. After irradiation, the relative expressions of HO-1 and Nrf2 proteins in nucleus were slightly increased ($P$<0.05), and the changes in cytoplasm were not obvious, whereas it was significantly increased in both nucleus and cytoplasm in the VU treatment groups. **Conclusions:** Anthocyanins extracted from VU could reduce apoptosis, stabilize cell membrane, and alleviate oxidant injury of mouse retinal photoreceptor 661W cells. The mechanism might be through activating Nrf2/HO-1 signal pathway and inducing HO-1 transcription and translation.

**KEYWORDS** *Vaccinium Uliginosum*, anthocyaninsm, photoreceptor, microwave, apoptosis, nuclear factor erythroid 2-related factor 2/heme oxygenase 1

The radiation damage to retina leads to retinal edema and hemorrhage, photoreceptor nucleus dissolution, and degeneration of outer segment.[1-4] Microwave radiation can increase reactive oxygen species (ROS) production by activating nicotinamide adenine dinucleotide (NADH) oxidase on cell membrane, leading to oxidative stress,[5] and excessive ROS can injure membrane structure,[6] resulting in retinal damage. Except for protection by apparatus, there is no effective drug treatments so far.

Anthocyanins, an effective active component of *Vaccinium Uliginosum* (VU), has good antioxidant and anti-apoptotic properties,[7] which can be achieved by activating nuclear factor erythroid 2-related factor 2/heme oxygenase 1(Nrf2/HO-1) signaling pathways.[8] Whether it can be used in prevention or treatment of microwave radiation damage in photosensitive cells has not been reported. In this study, we pretreated 661W cells of mouse retina using anthocyanins extracted from VU, and detected the changes of Nrf2

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and HO-1 mRNA expressions, to determine whether VU can alleviate the microwave radiation induced injury to photoreceptors.

## METHODS

### Cells, Drugs and Reagents

Photoreceptor 661W cells of mice were provided by ATCC Co. Ltd., USA. Dulbecco's Modified Eagle Medium (DMEM) cell culture medium and fetal bovine serum were provided by HyClone Co. Ltd., USA. Trypsin cell digestive solution was provided by GIBCO Co. Ltd., USA; malonaldehyde (MDA) and glutataione (GSH) test kits were provided by Abcam Co. Ltd. (Britain); superoxide dismutase (SOD) test kit was provided by Shanghai Beyotime Biotechnology Co. Ltd., China; enzymatic plate was provided by Nunc Co. Ltd., Denmark. Chromogenic substrate (TMB) was provided by Xinbosheng Biotechnology Co. Ltd. (China). Polyvinylidene fluoride (PVDF) transfer film was provided by Pall Co. Ltd. (USA); α tubulin monoclonal antibody and lamin B1(S-20) polyclonal antibody were provided by Santa Cruz Co. Ltd., USA. Other antibodies were provided by R&D Systems Co. Ltd., USA. Cytoplasm/nuclear protein extraction kits were provided by Fermentas Co. Ltd. (China). Pre-dye low molecular protein marker was provided by Promega Co. Ltd. (USA). Anthocyanins was extracted and provided by ultrasonic assisted-solvent extraction method by Schuool of Food Science and Nutrition Engineering of Agricultural University of China.

### Main Instruments

Desktop high-speed centrifuge (Type Biofuge pico) was provided by Heraeus Co. Ltd. (Germany). Biological inverted microscope (Type TMT2-21) was provided by Olympus Co. Ltd. (Japan). Decolorizing rocker (Type TS-100) was provided by Institute for Application of New Technologies (Beijing, China). Solid phase extraction column Sep-Pak C18 was provided by Waters Co. Ltd. (USA). Enzymatic Instrument (Type 318C) was provided by Shanghai Sanke Instrument Co. Ltd., China. Spectrophotometer was provided by JASCO (Japan). RQ-PCR instrument (Type IQ5), vertical electrophoresis cell and electrophoretic apparatus were provided by Bio-RAD Co. Ltd. (USA). The WKZ-Ⅱ microwave transmitter (frequency of microwave: 2,450 MHz) , provided by the Academy of Military Medical Sciences, China, was placed in the microwave radiation laboratory decorated with absorbing materials. The cells were placed in a radiation box made of plexiglass and the electromagnetic field was uniformly distributed.

### Preparation and Fractionation of Extracts

Fresh VU fruits was provided by Supply and Marketing Company of Da Hingganling Science and Technology Bureau, China and strictly followed the Chinese Pharmacopoeia, with cooperation of the School of Food Science and Nutritional Engineering, China Agricultural University, anthocyanins were extracted from VU by Fuleki extraction method.[8]

### Cell Grouping and Intervention

661W cells were divided into 6 groups, including control (661W cells without microwave radiation), model [661W cells radiated by microwave (30 mW/cm$^2$, 1 h)] and VU groups [661W cells pretreated with anthocyanins extracted from VU (25, 50, 100 and 200 μg/mL)[9,10] respectively for 48 h, and radiated by microwave 30 mW/cm$^2$, 1 h].

All cells were cultured in a $CO_2$ incubator of 37 ℃, 5% $CO_2$, 95% air, 100% humidity and received respective intervention. After radiation, cells in model and VU groups were cultured for another 6 h. Then the cell supernatant was harvested.

### Apoptosis Detection Under Fluorescence Microscope

Cells were observed under fluorescence microscope with Hoechst 33258 staining. Five visual fields were selected randomly in each group. Apoptosis index (AI) was calculated as the percentage of apoptotic cells in the total number of cells counted by Image processing software (Image pro plus 6.0).

### Cells Viability Detection

Cells were added with 1×MTT and cultured for 4 h, and the extract supernatant was added with 150 μL dimethyl sulfoxide for each test hole. After being shaked with a flat rocker, optical density was measured at the wavelength of 492 nm by the enzyme-reader. Cells viability was calculated according to the formula. The process was repeated 3 times.

### MDA, GSH and SOD Content Detection

MDA content was detected with MDA test kit. The supernatant was centrifuged at 4 ℃ 6,000 r/min for 5–10 min and MDA standard diluent was

prepared; 1: 2 continuously diluted supernatant of test cells was made and 10 μL of MDA color reagent stock solution was added to each MDA standard. After incubating at room temperature for 30 min, 40 μL of reaction solution was added and incubated for another 30 min. The absorbance at 695 nm was monitored.

GSH content was detected with GSH assay kit. The cells were washed with cold PBS and centrifuged. The standard diluent of GSH was added to supernatant and incubated at 37 ℃ for 60 min, keeping away from light, and the fluorescence intensity (ex/EM = 380/461 nm) was recorded every 2 min, and GSH content was calculated.

SOD activity was detected by SOD kit. The cells were washed with cold PBS. WST-8/enzyme working solution and reaction start-up working solution were prepared. The mixture was incubated at 37 ℃ for 30 min and its absorbance was measured at 450 nm. All experiments were repeated 3 times.

### Detection of mRNA Expressions of Nrf2 and HO-1 in 661W Cells by Real Time Quantitative PCR

RNAiso Plus was added to the cells. After centrifugation total RNA was extracted. Then reverse transcription reaction solution was prepared and cDNA was synthetized. The sequences of primers are shown in Table 1. Relative gene expressions of Nrf2 and HO-1 were analyzed by IQ 5 software, taking GAPDH as an internal reference.

**Table 1. Primers for RQ-PCR**

| Gene | | Sequence |
|---|---|---|
| Nrf2 | Forward | 5'- CACATCCAGACAGACACCAGT -3' |
| | Reverse | 5'-CTACAAATGGGAATGTCTCTGC-3' |
| HO-1 | Forward | 5'- CAGAAGGGTCAGGTGTCCAG -3' |
| | Reverse | 5'- GAAGGCCATGTCCTGCTCTA -3' |
| GAPDH | Forward | 5'-GCCAGCCTCGTCTCATAGACA-3' |
| | Reverse | 5'-AGAGAAGGCAGCCCTGGTAAC-3' |

### Detection of HO-1 and Nrf2 Protein Expression in 661W Cells by Western Blot

The Western hybridization signal band on X-ray film was scanned by gray scale. Protein expressions of HO-1 and GAPDH were analyzed by Image Pro Plus 6.0 Image Analysis Software. The relative expression of HO-1 protein was obtained by calculating the ratio of HO-1 to GAPDH (presented in relative units). The relative expression of Nrf2 protein in nuclear protein was obtained by calculating the ratio of Nrf2 to lamin B1, and the relative expression of Nrf2 in cytoplasmic protein was obtained by the ratio of Nrf2 to Tubulin (presented in relative units).

### Statistical Analysis

All data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and analyzed with SPSS 21.0 software. ANOVA was applied for data with normal distribution and homogeneity of variance. One-way ANOVA was applied for comparison of measurement data among groups. If the difference was significant, the Dunnett test was further used to test the significance of intergroup differences. Linear regression and correlation analysis were used to test the dose-dependent manner. $P<0.05$ was considered statistically significant.

## RESULTS

### Anthocyanins in VU

High performance liquid chromatography (HPLC) and total ion flow diagram of extracted anthocyanins are shown in Figure 1.

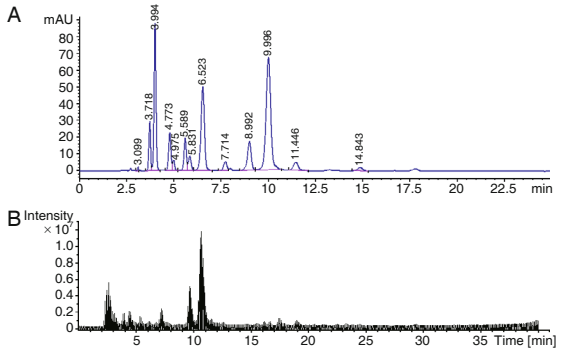


**Figure 1. High Performance Liquid Chromatography (HPLC, A) and Total Ion Flow Diagram (B) of Extracted Anthocyanins**

### VU Decreases Apoptosis of 661W Cells Injured by Microwave Irradiation

There appeared a lot of apoptotic cells in the model group 6 h after microwave irradiation. After VU pretreatment, apoptosis was significantly improved (Figure 2A). Dunnett method showed that AI was higher in the model group, and was decreased obviously in 4 VU pretreated groups (all $P<0.05$). Linear regression analysis showed that the AI was decreased in a dose-dependent manner by VU treatment ($r=0.8419$, $P<0.05$, Figure 2B).

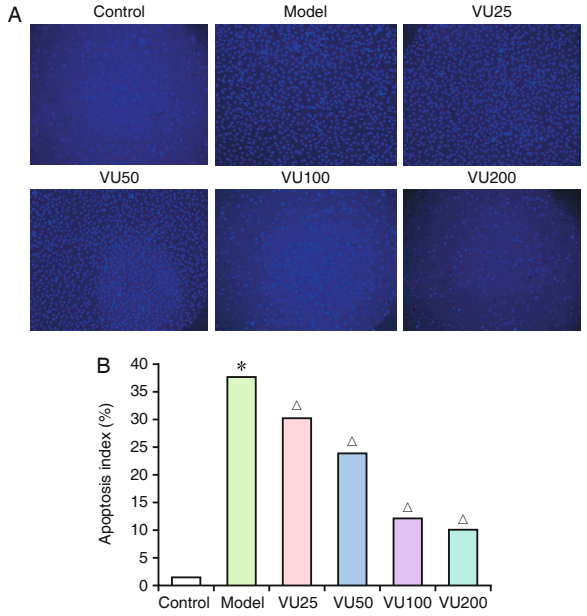


**Figure 2. Effect of VU on Apoptosis of 661W Cells Injured by Microwave Radiation**

Notes: A: Cells were harvested and stained with Hoechst 33258. The staining showed strong blue fluorescence in apoptotic cells, and the normal cells only showed weak fluorescence, and the dead cells were not stained (×200). B: *$P$<0.05 *vs*. control group; △$P$<0.05 *vs*. model group

## Effect of VU on Cells Viability of 661W Cells Damaged by Microwave Irradiation

One-way ANOVA showed that significant difference of cells viability exists among all the groups ($F$=70.91, $P$<0.05). Dunnett method showed that cells viability in the model group were lower than the control group ($P$<0.05). As shown in Figure 3, VU100 and VU200 pretreatment significantly increase the cell viability compared with the model group ($P$<0.05).

## Effect of VU on MDA, GSH Content and SOD Activity of 661W Cells Injured by Microwave Irradiation

The content of MDA in the model group was significantly higher than the control group, and it was lowered in the 4 VU-treated groups (all $P$<0.05).

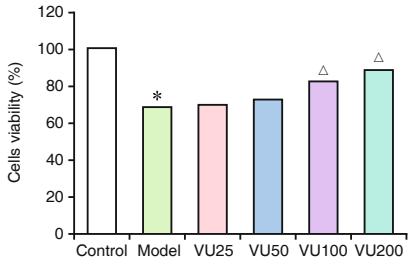


**Figure 3. Effect of Extracts from VU on Cells Viability of 661W Cells Injured by Microwave Radiation**

Notes: *$P$<0.05 *vs*. control group; △$P$<0.05 *vs*. model group

According to linear regression analysis, there was no dose-dependent effect in the 4 VU pretreated groups ($r$= 0.211, $P$>0.05; Figure 4A). The GSH content of 661W cells was significantly lower in the model group compared with the control group ($P$<0.05), and significantly higher in the 4 VU pretreated groups compared with the model group ($P$<0.05, Figure 4B). The SOD activity of 661W cells was significantly lower in the model group than the control group, and it was significantly higher in the VU pretreated groups (except for VU25, $P$<0.05; Figure 4C). There was no significant difference among VU groups in the MDA, GSH content and SOD activity ($P$>0.05).

Statistically significant difference can be seen only after the concentration of VU rose to 100 μg/mL. Meanwhile, there was significant difference among VU100 and model groups in MDA, GSH and SOD levels ($P$<0.05). According to the principle of the lowest agent, 100 μg/mL was chosen for further mechanism tests.

## mRNA Expressions of Nrf2 and HO-1 in 661W Cells

There were significant differences among groups in the relative mRNA expressions of Nrf2 and HO-1 (all $P$<0.05). Further Dunnett test showed that HO-1 mRNA expression was higher in the model group, and increased significantly in VU100 pretreated group (all

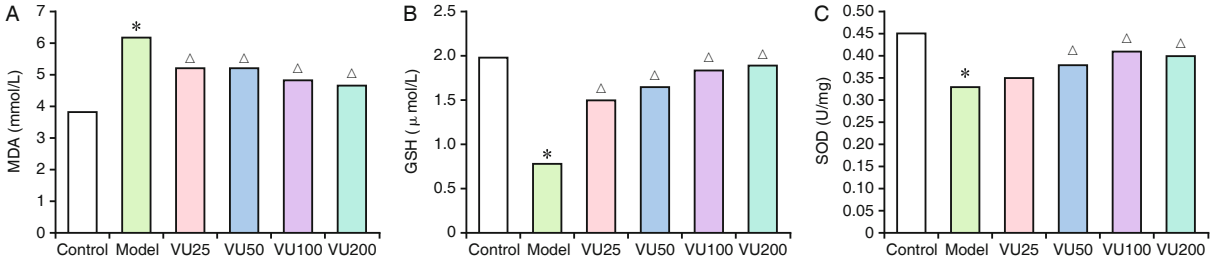


**Figure 4. Effect of Extracts from VU on MDA (A), GSH (B) and SOD Activity (C) of 661W Cells Injured by Microwave Radiation**

Notes: *$P$<0.05 *vs*. control group; △$P$<0.05 *vs*. model group

**Table 2. Relative mRNA Expressions of Nrf2 and HO-1 in 661W Cells among Groups**

| Group | Nrf2/GAPDH | HO-1/GAPDH |
|---|---|---|
| Control | 1.05 ± 0.14 | 1.01 ± 0.24 |
| Model | 1.54 ± 0.20* | 1.47 ± 0.14* |
| VU100 | 6.77 ± 1.21△ | 5.65 ± 1.00△ |

Notes: *$P$<0.05 *vs*. control group; △$P$<0.05 *vs*. model group

$P$<0.05; Table 2, Figure 5A).

## Protein Expressions of HO-1 and Nrf2 in 661W Cells

Figure 5B shows the protein expression of Nrf2 from nucleus and cytoplasm. There was no significant difference in cytoplasm between model and control groups ($P$>0.05), and the protein expression level of Nrf2 significantly increased in the VU100 pretreated group ($P$<0.05). Nrf2 protein in nucleus increased slightly after irradiation, and significantly increased after pretreatment of 100 μg/mL VU (Figure 5C).

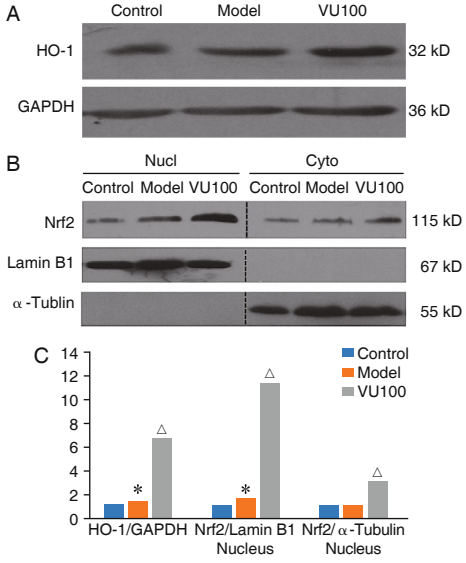


**Figure 5. HO-1 and Nrf2 Protein Expressions by Western Blot**

Notes: *$P$<0.05 *vs*. control group; △$P$<0.05 *vs*. model group

## DISCUSSION

Microwave is a physical phenomenon widely existing in the environment. In recent years, microwave emitted by wireless devices has increased rapidly in the world.[11] The mechanism of microwave injury to retina is mainly due to imbalance between oxidation and antioxidant, which produces many free radicals, leading to lipid peroxidation damage and cell apoptosis.[12] At present, there is no effective solution. In this experiment, AI and MDA content of retinal photoreceptors in the model group significantly increased compared with the control group. MDA is one of the important markers to evaluate lipid peroxidation, and an important detection index to evaluate cell membrane stability and cell oxidative damage.[13] Therefore, it is verified that the retinal photoreceptors have cell damage after microwave radiation, and lipid peroxidation occurs in the process of damage.

The antioxidant effect of anthocyanins has been widely verified. It can block the chain reaction of free radicals by stabilizing semiquinone, and reduce oxidative damage.[14] It is widely used in improving immunity, nourishing essence, and refreshing and delaying aging disease, etc.,[15] however, it has not been applied in the microwave prevention and treatment of retina damage.

In terms of damage mechanism, retinal light damage is similar to microwave damage, mainly due to the destruction of oxygen balance caused by lipid peroxidation.[16] At present, anthocyanins have been widely used, and its protective effects on retinal light damage has been confirmed.[17] Light damage is called "light poison" in traditional medicine, which may cause Gan (Liver) and Shen (Kidney) deficiency, as well as deficiency of qi and blood.

VU is a deciduous shrub of Rhododendron family and Vaccinium genus. According to study of Human Nutrition Center, the Department of Agriculture of the United States, vaccinium has the best antioxidant capacity among 282 kinds of fruits and vegetables, because it has the most abundant anthocyanins.[18]

In this study, we found that apoptosis in the VU pretreatment groups was decreased compared with model group, with a dose-dependent manner, indicating that VU can effectively reduce the microwave radiation injury. SOD and MDA are important antioxidant enzymes *in vivo*. In this study, we found that the activity of SOD and MDA in 661W cells decreased after microwave radiation injury. They increased in all VU pretreatment groups compared with model group, indicating that VU extracts can effectively reduce the damage and protect the stability of cell membrane.

GSH widely exists in animals and plants, as an

antioxidant being capable of scavenging free radicals, its main physiological function is to remove free radicals in the human body, and protects many thiols in proteins and enzymes.[19] It was found in our study that the GSH content of 661W cells decreased after microwave irradiation, and was significantly increased in the VU pretreatment groups, indicating that VU has a protective effect on oxidative stress injury induced by microwave radiation.

In recent years, Nrf2 induced HO-1 expression has been considered as one of the most important mechanisms of intracellular antioxidant stress.[20] Therefore, it is a research direction to study how to increase the expression of downstream products including HO-1 after microwave radiation injury by increasing Nrf2 expression.

Under normal circumstances, Nrf2 and its inhibitor protein Keap-1 are connected in the cytoplasm inactively. In case of oxidative stress, Nrf2 dissociates from Keap-1 and enters the nucleus. After binding with Maf protein, Nrf2 connects with antioxidant response element (ARE) to initiate transcription of antioxidant gene HO-1.[21,22] HO-1, as an antioxidant enzyme, can not only prevent free heme from participating in the oxidation reaction, but also inhibit apoptosis, exert anti-inflammatory and vasodilator effects,[23] which is one of the most important endogenous protection systems in the body.

This study showed that mRNA expressions of Nrf2 and HO-1 increased slightly after microwave irradiation, and significantly elevated after VU pretreatment. In view of the different position of Nrf2 before and after being activated, to certificate the signaling pathway, the protein expression of Nrf2 in nucleus and cytoplasm was detected respectively. The results showed that Nrf2 protein in nucleus increased slightly at 6 h after microwave irradiation, but the change in the cytoplasmic protein was not statistically significant. However, VU significantly increased the nuclear Nrf2 protein content and only slightly increased the cytoplasmic Nrf2 protein. This provided further evidence for Nrf2/HO-1 signaling pathway participating in the 661W cells protection by VU. Being activated by VU, Nrf2 is transferred into nucleus and Nrf2/HO-1 signaling pathway works. As one of the downstream products, HO-1 is transcribed and translated, so that cells are protected against oxidative damage induced by microwave radiation.

Through these results, we can preliminarily confirm that anthocyanins extracted from VU reduces apoptosis of 661W cells induced by microwave, and the stability of cell membrane can be enhanced, and the antioxidant capacity be improved.

## Conflict of Interest

The authors declare that they have no conflict of interests.

## Author Contributions

Yin L contributed to the conception of the study; Yin L and Fan SJ conducted the experiment and wrote the manuscript; Zhang MN analyzed the data. All author read the paper and approved the final version for publication.

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