Free Radicals and Antioxidants., 2023; 13(2):60-66.
https://www.antiox.org

Original Article

Orientin Protects BV-2 Microglial Cells against Hypoxia Reoxygenation Injury through Inhibiting Oxidative Stress

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ABSTRACT

Background: Oxidative stress is one of the pivotal pathogenesis of ischemic stroke. Orientin, a compound extracted from plants, has been previously reported to have antioxidant activity that was exploited in various diseases. The objective of this study was to focus on the potential therapeutic effects of orientin on cobalt chloride ($CoCl_2$)-induced Hypoxia Reoxygenation (HR) injury in BV-2 microglial cells and to explore the possible mechanisms. **Materials and Methods:** The optimum protective condition of orientin on BV-2 microglial cells after HR injury was evaluated by the viability of cells measured by Cell Counting Kit-8 (CCK-8). Afterwards, functions of Superoxide Dismutase (SOD) and Catalase (CAT) as well as the content of Glutathione Peroxidase (GSH-PX) were measured by corresponding kits. Activation of Nrf2 pathway was assayed by western blotting. **Results:** Our study demonstrates that the viability of HR injured BV-2 microglial cells was improved significantly after incubation with orientin (40~100 µg/mL) for 8 hr. Furthermore, orientin was found to increase the nuclear translocation of Nrf2 protein in BV-2 microglial cells and was shown to promote antioxidant enzyme activities and enhance the expression of HO-1 and NQO1. **Conclusion:** Taken together, Results of our study indicate that orientin protect BV-2 microglial cells against HR injury induced by $CoCl_2$, and this protection is associated with the inhibition of oxidative stress through the activation of the Nrf2 signaling pathway.

Keywords: Oarientin, Oxidative stress, Hypoxia reperfusion injury, BV-2 microglial cells, Nrf2.

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Received: 21-08-2023;
Revised: 03-09-2023;
Accepted: 30-09-2023.

INTRODUCTION

As one of the major diseases that threaten human health, stroke has the characteristics of high incidence and high mortality; and about 85% are ischemia stroke which seriously affects the life quality of the patient and their family members.[1,2] Although different mechanisms are involved in the causes of ischemia reperfusion injury,[3] increasing evidence indicates that oxidative damage induced by oxidative stress plays a key role in ischemic stroke injury.[4,5] Indeed, when human body is subjected to various harmful stimuli, such as ischemic or hypoxic, excessive Reactive Oxygen Species (ROS) in the body or tissue cells are produced, which breaks the balance of oxidant and antioxidant, thus leading to tissue damage.[6,7] Therefore, inhibition of oxidative stress or modulating their signaling pathways could be a potential approach for fighting ischemic stroke. In particular, endogenous activation of the Nrf2 signaling pathway is of vital importance in mitigating cellular oxidative stress.[8,9]

Here we investigated the protective effects of orientin derived from *Cyperus esculentus* L. (CEL) on cellular hypoxic injury induced by cobalt chloride ($CoCl_2$). Orientin belongs to flavonoid family and is the major ingredient that can be extracted from a variety of plants. It has cardioprotective and neuroprotective effects, and also possesses anti-viral, anti-bacterial and anti-inflammation properties.[10] Several studies have demonstrated that orientin also possesses significant antioxidant activity that could be used to scavenge ROS when applied pharmacologically.[11,12] Additionally, orientin has been reported to have anti-thrombotic effect and

DOI: 10.5530/fra.2023.2.11

may do so through the prevention of thrombus formation.[13-15] Furthermore, numerous studies have also been focused on the protective effect of orientin on myocardial ischemic injury.[16] For example, Liu *et al.*[17] have reported that orientin could protect cardiac cells against injury-induced by hypoxia through inhibition of autophagy.

Our group[18] previously determined that among the CEL extracts, the fraction with the strongest antioxidant activity was the 30% ethanol elution fraction of the ethanol extract from the stems and leaves. Further isolation and purification obtained four monomers. Compound 1 was 7-hydroxy-6-methoxy-coumarin, compounds 2 and 3 were Isoorientin, and compound 4 was orientin (Figure 1A). More analysis indicated that compound 4 (Orientin) was the main component among the four monomeric compounds, accounting for 65.4% (mass percentage). Indeed, orientin, as a natural flavonoid compound[19] is the main active ingredient of various plants and is widely distributed in leaves, stems, seeds and fruits of dozens of plants including buttercup, Polygonum, Polyphylla, Palmetto, leguminosae, Labiaceae, and chrysanthemum 10. Modern pharmacological studies have shown that orientin had a wide range of pharmacological activities such as anti-inflammatory, anti-tumor, cardioprotective, neuroprotective and analgesic[20] It is worth mentioning that the most significant pharmacological activity of orientin is its anti-inflammatory activity *in vitro* and *in vivo*.[21]

While orientin is known to exert neuroprotection in rats following cerebral ischemia reperfusion injury via promoting autophagy,[22] few studies have examined orientin's inhibitory effects on oxidative stress and the underlying signaling pathways, in particular, in microglia. Microglia can be activated by Hypoxia Reoxygenation (HR) injury and may play a dual role of either protection or damage in the central nervous system. It would be beneficial if microglia's protection capacity is enhanced while microglia's damaging effect is minimized in cerebral ischemia.[23,24] Thus, the present study was carried out to investigate the ameliorating effect of orientin on BV-2 microglial cells after HR injury and the potential underlying mechanisms. In the present study, cell hypoxic injury was induced by $CoCl_2$.

MATERIALS AND METHODS

Materials and Reagents

Orientin and $CoCl_2$ were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Orientin was dissolved in Dimethyl Sulfoxide (DMSO), and the final concentration of DMSO was 0.2%. This solution was further diluted in Dulbecco's Modified Eagle's Medium (DMEM) to reach different concentrations of orientin (40, 80, 100, 200, 400 μg/mL). Edaravone was used as a positive control and was purchased from Beijing Solarbio Technology Co., Ltd. (Beijing, China). DMSO was obtained from Sigma (St. Louis, MO, USA). DMEM, Phosphate Buffered Saline (PBS) and penicillin and streptomycin were all from Hyclone (Logan, Utah, USA). Fetal Bovine Serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). CCK-8 was obtained from Wuhan Boster Biological Technology., Ltd. (Wuhan, China).

BV-2 microglia culture and HR injury treatment protocol

BV-2 microglial microglia were contributed by Professor Suhua Qi from Xuzhou Medical University and cultured in DMEM supplemented with 10% FBS. HR injury model was established by $CoCl_2$, which is usually used as an *in vitro* inducer for cellular hypoxia.[25,26] Basically, BV-2 microglial cells were treated with different dose of $CoCl_2$ for 16 hr[27] and then re-incubated with DMEM for various reperfusion time points (0, 4, 8, 12, 24 hr) to establish the HR injury model. Based on our pilot studies, 40~400 μg/mL of orientin were used for reperfusion of BV-2 microglial cells that were treated with 200 μM of $CoCl_2$ for 16 hr. We finally selected the optimum dose of $CoCl_2$ (200 μM) and orientin reperfusion for 8 hr as HR injury model in subsequent experiments. The complete experimental design is shown in Figure 1. In addition, BV-2 microglial cells were then seeded into electronic 8-well plates (1×10^5 cells/well) to further investigate the effects of orientin and positive control reagent on the viability of cells by quantifying cell proliferation in a label-free, real-time manner with the support of the RTCA iCELLigence instrument (ACEA Biosciences, USA). The electronic 8-well plates with cells

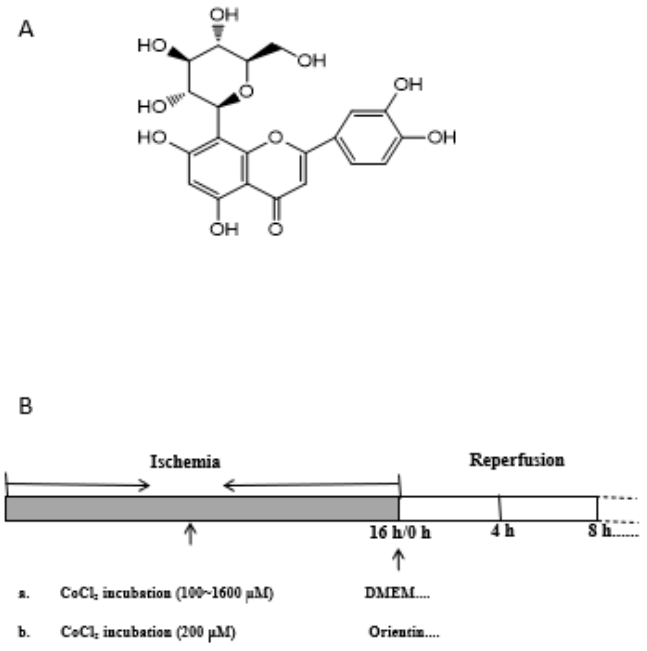

Figure 1: (A) Chemical structure of orientin. (B) Diagrammatic sketch of the experimental design. (A) $CoCl_2$ induction of hypoxic injury in BV-2 MICROGLIAL cells; (B) Protective effect of orientin on BV-2 MICROGLIAL cells after hypoxic reperfusion (HR) injury.

were then placed in a standard CO_2 cell culture incubator for further analysis as described below.

Cell viability and death

BV-2 microglial cell viability was evaluated by using CCK-8 kit. According to the instruction manual, culture medium in 8-well plates was removed and replaced with 100 µL PBS containing 10% CCK reagent for 0.5-1 hr after experimental treatments. Then, the absorbance values of each well were measured at 450 nm under Microplate reader (Beijing Safe Heart Technology Co. Ltd.) and the cell viability of the test groups was presented as a percentage of the mean absorbance of the control group.

Antioxidant enzyme assays

The HR injury model of BV-2 microglial cells was established as described above under 2.2. For investigation of the antioxidant capacity of orientin on HR injured BV-2 microglial cells, the control group, model group, orientin groups and positive group (200 µg/mL of edaravone) were examined. All groups of BV-2 microglial cells were plated into 6-well plates (1×10^5 cells/well) for 24 hr incubation. Next, the cells, except the control group, were exposed to $CoCl_2$ (200 µM) for 16 hr, and followed by treatment with different doses of orientin or edaravone for 8 hr while pure DMEM was used for the model group. The levels of CAT and SOD as well as the content of GSH-PX were measured using corresponding assay kits according to manufacturers' instructions. All the assay kits were purchased from Nanjing Jiancheng Biotechnology Co. Ltd. (Nanjing, China). SOD, CAT, and MDA were quantified using a microplate reader.

Western blot analysis

The group design of the cell experiment is also as described above. Western blot was performed on 12% resolving Sodium Dodecyl Sulfate (SDS)-PAGE gel according to Kim[28] with slight modifications. Total protein was obtained by RIPA lysis buffer (Solarbio, China) while cytoplasmic and nuclear proteins were prepared using extraction kit (Boster, China) according to the instructions. Following SDS-PAGE, proteins were transferred onto Polyvinylidene Difluoride (PVDF) membrane that was then blocked for 2 hr in TBST with 5% skim milk. The membranes were incubated overnight at 4℃ with primary antibody dilution by TBST (β-actin, 1:1000, Abcam, USA; Nrf2, HO-1, 1:8000, Abcam, USA; NQO1, 1:1000, Abcam, USA). Horseradish peroxidase-conjugated secondary antibodies were then applied, and the blot was visualized using ECL detection kit. The intensity of each blot was quantified by Image-J software (USA).

Statistical analysis

All data were expressed as mean±SD and statistical analysis was conducted with SPSS 19.0 software (SPSS, Chicago, USA). A value of $p<0.05$ was considered significant.

RESULTS

Orientin protects BV-2 microglial cells against $CoCl_2$-induced HR injury

We found that the viability of BV-2 microglial cells gradually decreased when $CoCl_2$ dose and reperfusion time increased (Figure 2 A~E) which reflected an obvious dose and time dependent effect. After $CoCl_2$ treatment for 16 hr at 200 µM, BV-2 microglial cell viability rate were 61.67±4.396%, 58.19±0.105%, 69.81±4.525%, 68.52±4.514%, 56.52±3.236%, respectively, at different reperfusion time (0, 4, 8, 12, 24 hr) and the average rate was 62.94%. Therefore, we choose 200 µM of $CoCl_2$ as the suitable dose for subsequent studies under our experimental conditions, under which BV-2 microglial cells were damaged in the absence of a large number of deaths. Results in Figure 3 A-C showed that orientin could improve the BV-2 microglial cell viability rate at a particular point. When reperfusion was given for 4 hr or 24 hr, we found that the dosage of 40 µg/mL orientin was capable of protecting BV-2 miroglial cells against HR injury ($p>0.05$). However, when the reperfusion dosage was set at 40, 80 and 100 µg/mL and the reperfusion length was set for 8 hr, the cells' average viability was 68.30±9.01%, 68.99±9.83%, 78.81±6.30% ($p<0.05$), respectively, which were all significantly increased when compared with the model group (65.79±7.86%). In addition, the positive group (200 µg/mL of edaravone) also exhibited a significant increase ($p<0.01$) in cell viability (Figure 3D). Therefore, the dosage of 200 µg/mL was used in subsequent studies.

From the real-time dynamic monitoring curve of viability of HR injured BV-2 microglial cells (Figure 4), we observed that all groups of cells showed a similar growth trend in the initial 0-22 hr. At this stage, the cells were present in a Logarithmic growth and the density rapidly increased. Then at the hypoxic stage (200 µM $CoCl_2$ incubation for 16 hr), the curve of the experiment groups decreased quickly due to the damage by $CoCl_2$, while control group incubated with serum-free DMEM was relatively flat and basically maintained the original cell density. Finally, BV-2 microglial cells were incubated with orientin (40, 80 µg/mL) and edaravone (200 µg/mL) for a reperfusion stage while model group was incubated with DMEM. The growth curve of the orientin groups and positive group increased, indicating that orientin could improve the BV-2 microglial cell viability rate and such a rate was higher than that of the model group. In conclusion, these results showed that orientin could significantly improve the viability of HR injured BV-2 microglial cells. We therefore further investigated the protection mechanisms.

Orientin improve the activity of antioxidant enzyme in BV-2 microglial cells after HR injury

When compared with control groups, the activities of SOD, GSH-PX and CAT significantly decreased in model groups, indicating that BV-2 microglial cells were suffered from severe

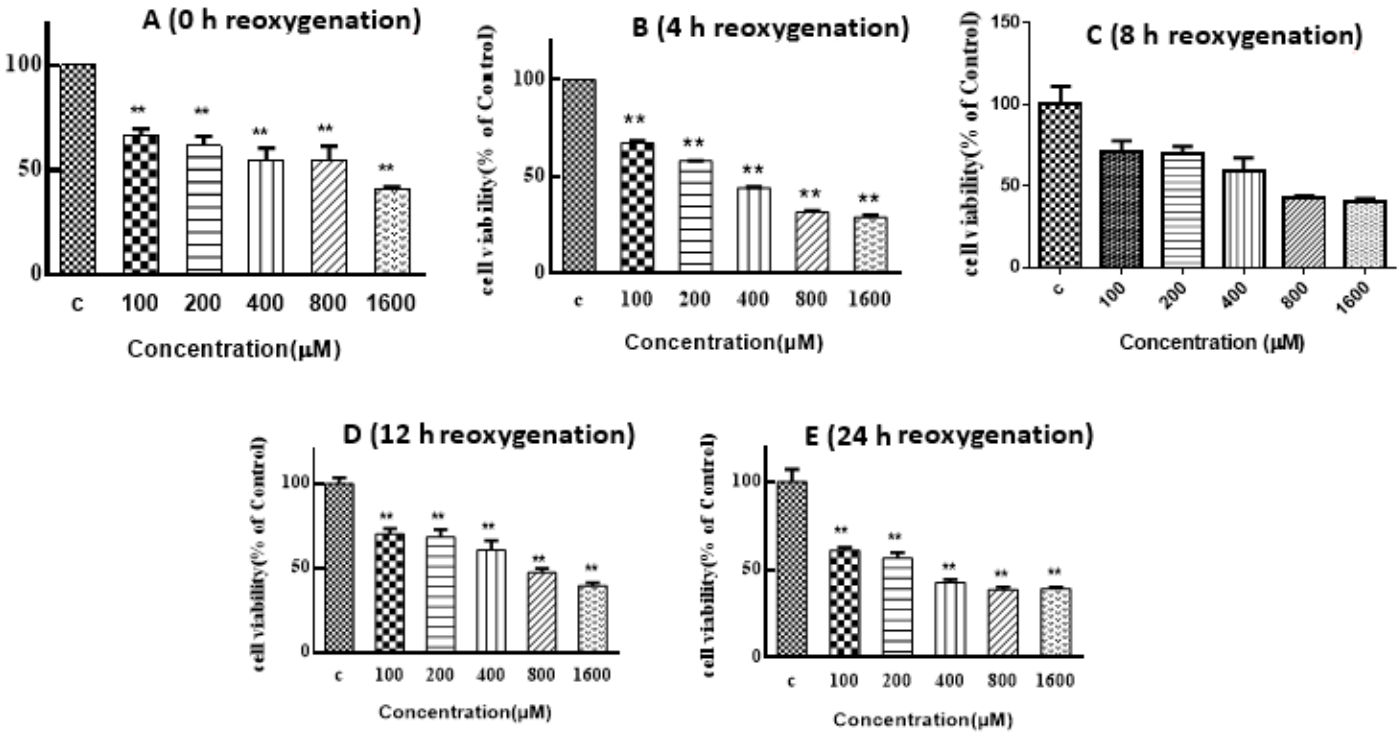

Figure 2: Viability of BV-2 MICROGLIAL after incubation with $CoCl_2$ for 16 hr and reperfusion for different hours with DMEM. (A) Reperfusion for 0 h; (B) Reperfusion for 4 hr; (C) Reperfusion for 8 hr; (D) Reperfusion for 12 hr; (E) Reperfusion for 24 hr. Note: *$p < 0.05$, **$p < 0.01$ compared with control.

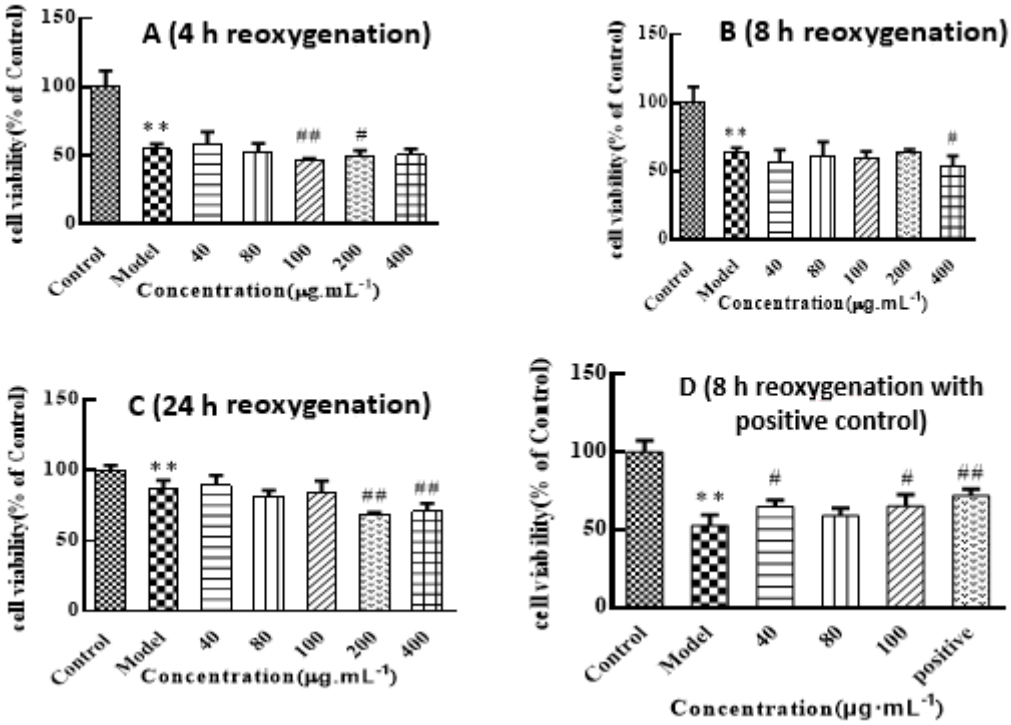

Figure 3: Protective effect of orientin on BV-2 MICROGLIAL cells against $CoCl_2$-induced HR injury. (A) Orientin reperfusion for 4 hr; (B) Orientin reperfusion for 8 hr; (C) Orientin reperfusion for 24 hr; (D) Orientin reperfusion for 8 hr (containing positive group). Note: Compared with control,$^{*}p<0.05$，$^{**}p<0.01$; Compared with model, $^{\#}p<0.05$，$^{\#}p<0.01$.

oxidative damage after HR injury (Figure 5 A~C). Both 80 and 100 μg/mL of orientin could remarkably improve (p<0.01) the SOD activity (10.225±0.18, 9.242±0.20 *U*/mL), approximately to that of the positive group (10.716±0.10 U/mL) (Figure 5A). Besides, the GSH-PX activity in HR injured BV-2 microglial cells was significantly enhanced with the increasing concentration of orientin (40, 80, 100 μg/mL) in a dose-dependent manner. The content of intracellular CAT also increased dramatically (p<0.01) after incubation with orientin. When the concentration of orientin was 80 μg/mL, the CAT activity was 0.981±0.072 U/mL, which was 9.25 times of the model group, and higher than that of the control group and of the positive group. These results indicate that HR injured BV-2 microglial cells exposed to orientin (40, 80, 100 μg/mL) for 8 hr obviously exhibited increased activities of antioxidant enzymes to attenuate oxidative damage.

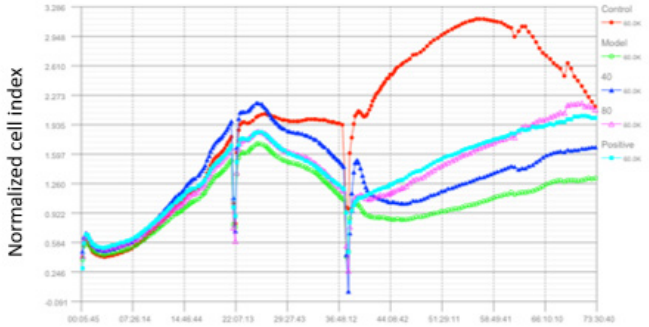

Figure 4: Real-time dynamic monitoring curve of viability of HR injured BV-2 MICROGLIAL cells incubated with orientin for 8 hr.

Orientin treatment up-regulates the Nrf2 antioxidative signaling pathways in BV-2 microglial cells after HR injury.

The Nrf2 signal pathway is widely considered as the mechanism of anti-oxidative stress. Expression of down-stream phase Ⅱ metabolic enzymes and antioxidant enzymes, such as quinone oxidoreductase-1 (NQO1) and Heme Oxygenase-1 (HO-1) could be initiated to protect cells against oxidative damage when Nrf2 signal pathway was activated.[29] In the present study, BV-2 microglial cells were pretreated with $CoCl_2$ (200 μM) for 16 hr and further incubated with orientin (40 and 80 μg/mL) or positive regents (edaravone 200 μg/mL) for 8 hr. This was followed by determining the expression of HO-1 protein, NQO1 and Nrf2 by western blotting. Results indicate that exposure to 40 μg/mL of orientin could efficiently induce the expression of HO-1 and NQO1 protein in BV-2 microglial cells (p<0.01), which was almost consistent with that of the positive group and those having higher than 80 μg/mL of orientin (Figure 6 A and B). In addition, as shown in Figure 6C and D, levels of Nrf2 increased dramatically in the nuclear (almost 1.3 folds of that of model group, p< 0.01) after incubation with 40 μg/mL of orientin. This nuclear increase was concurrent with a decrease of cytoplasmic Nrf2 (about 46.6% of model group, p<0.05). These results strongly indicate that orientin could also significantly protect BV-2 microglial cells against oxidative damage through the signaling pathway of Nrf2.

DISCUSSION

Oxidative damage occurs in cells when excessive ROS are generated by external stimulus like ischemic injury. Normally, in response to oxidative stress, tissues can mobilize free radical scavenger systems to mitigate damage by ROS. However, the function of these scavengers could be attenuated in ischemic tissue because of cell injury and blood reflow. In particular, ischemic

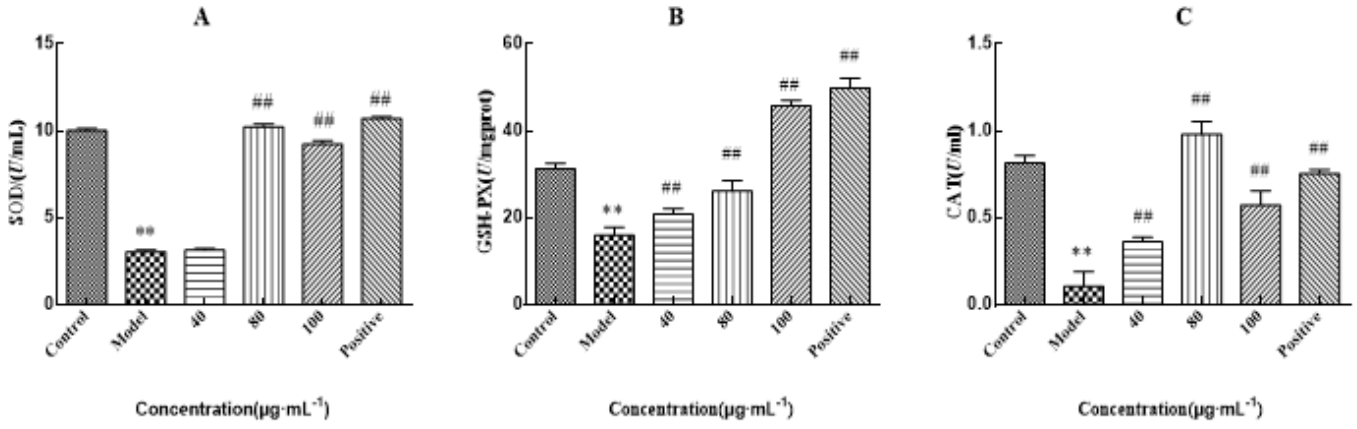

Figure 5: Effect of Orientin on antioxidant enzyme content in BV-2 MICROGLIAL cells after HR injury. (A) SOD activity; (B) GSH-PX activity; (C) CAT activity. Note: Compared with control,$^{*}p$<0.05，$^{**}p$<0.01，Compared with model; $^{\#}p$<0.05，$^{\#\#}p$<0.01.

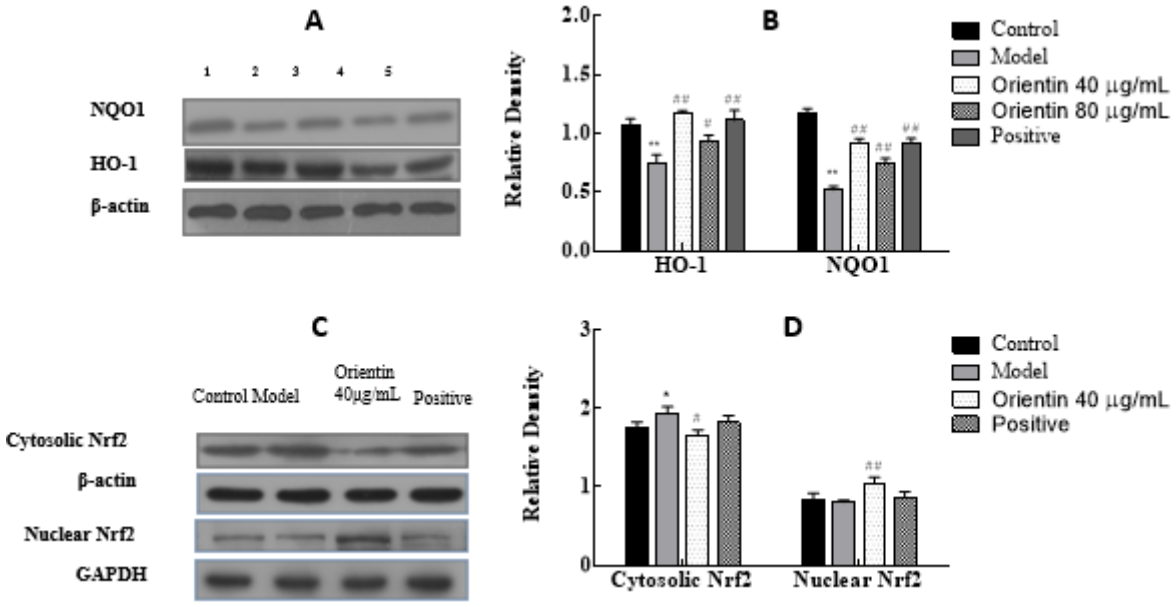

Figure 6: Effects of orientin on HO-1, NQO1 and Nrf2 expression in BV-2 MICROGLIAL cells after IHR injury. (A) Cells were treated with various doses of Orientin and positive regents (1-Control, 2-Model, 3-Orientin 40 μg/mL, 4-Orientin 80 μg/mL, 5-Positive); (B) Quantification of HO-1 and NQO1protein expression was performed by densitometric analysis and normalized to β-actin. Representative graph (C) and quantitative data (D) on the expression of Nrf2 in the Cytosolic and in the nuclear. Note: Compared with control,$^{*}p<0.05$，$^{**}p<0.01$，Compared with model, $^{\#}p<0.05$，$^{\#\#}p<0.01$.

tissue can be further damaged by the free radicals generated upon reperfusion due to sudden oxygen resupply.[30,31]

The purpose of this study was to explore the protective effects of orientin on hypoxia-induced damage to BV-2 microglial cells. Our data demonstrated that orientin could protect BV-2 microglial cells against $CoCl_2$-induced HR injury by promoting the content of antioxidant enzymes. Furthermore, we found that orientin could also alleviate oxidative damage via up-regulating the activities of Nrf2 signaling pathway.

Orientin is an antioxidant compound that has been attracting increasing attention in recent years. This compound can also be extracted with a good yield from multiple natural plants[11,32] We herein investigated the protective effects of orientin using BV-2 microglial microglia as an HR injury system. In particular, we explored the Nrf2 signaling pathway and wanted to know whether this signaling pathway could be activated by orientin in a cellular model of hypoxic injury. Nrf2 is a crucial factor in Keap1-Nrf2/Antioxidative Response Element (ARE) signaling pathway, and the protein normally is in an inactive state due to binding by its inhibitor Keap1 in the cytoplasm.[29,33] Usually, inactive Nrf2 protein in normal cells will be rapidly degraded with a half-life ($T_{1/2}$) of only 10 to 30 min.[34] Once challenged with oxidative stress, Nrf2 can dissociate from Keap1 and translocate into the nucleus. After becoming a heterodimer with small Maf protein within the nucleus, Nrf2 binds ARE to activate target gene expression. In the present study, we demonstrate that orientin is capable of activating the Nrf2 signaling pathway (Figure 6) in that there was an increase in NQO1 and HO-1 expressions due to Nrf2 translocation from cytosol to nuclear.

In summary, our results revealed the inhibition of oxidative stress by orientin in an *in vitro* HR injury model of microglial, which is often used as a pharmacological basis for testing the protective effects of compounds or drugs on ischemic stroke. However, there are still several limitations in this study, which called for further investigations. The major limitation is that we only explored the effect of post-conditioning of orientin on BV-2 microglial cells; the preventive effect of pre-conditioning of orientin on BV-2 microglial cells thus will need further investigation. Nonetheless, our study points to the potential application of orientin as a potent antioxidant for fighting stroke injury.

CONCLUSION

We have demonstrated in the present study that orientin protect BV-2 microglial cells against HR injury induced by CoCl2, and this protection is associated with the inhibition of oxidative stress through the activation of the Nrf2 signaling pathway.

ACKNOWLEDGEMENT

This work was supported in part by National Science Foundation of China (No. 31770385, Fund manager: Siqun Jing), Key research project of universities in Guangdong Province (No. 2019KZDXM053, Fund manager: Siqun Jing), 2021 Guangdong Natural Science Foundation project(2021A1515011262) and Grants of the National Natural Science Foundation of China (No. 81671164, Fund manager: Suhua Qi).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ARE: Antioxidant response element; **$CoCl_2$:** Cobalt chloride; **HR:** Hypoxia reperfusion; **CCK-8:** Cell Counting Kit-8; **SOD:** Superoxide dismutase; **CAT:** Catalase; **GSH-PX:** Glutathione peroxidase; **ROS:** Reactive oxygen species; **NQO1**: Quinone oxidoreductase-1; **HO-1:** Heme oxygenase-1; **DMSO:** Dimethyl sulfoxide; **DMEM:** Dulbecco's modified Eagle's medium; **PBS:** Phosphate buffered saline; **FBS:** Fetal bovine serum; **SDS-PAGE**: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; **PVDF:** Polyvinylidene difluoride.

AUTHOR CONTRIBUTION

HZ, JZ, and GL did experiments and were involved in data collection. SJ, YT, JC, SQ and YGL and LJY designed the experiments, interpreted data, and wrote, reviewed and edited the manuscript.

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Cite this article: Jing S, Tu Y, Zhai H, Zhang J, Liu G, Cheng J, *et al*. Orientin Protects BV-2 Microglial Cells against Hypoxia Reoxygenation Injury through Inhibiting Oxidative Stress. Free Radicals and Antioxidants. 2023;13(2):60-6.