Neuroprotective Effects of Propylgallate Against Oxidative Stress in Retinal Ganglion Cells

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Research Article

Keywords: Diabetic retinopathy, oxidative stress model, Propylgallate, neuroprotective effects, Apoptosis PCR array

Posted Date: February 23rd, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-870585/v2>

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Abstract

Background: Diabetic retinopathy is a group of eye diseases which result in damage to the optic nerve and vision loss, it has seriously affect peoples' health. The purpose of this study is to contrast the neuroprotective effects of curcumin, gastrodin, propylgallate, adenosine. At the same time, we preliminarily explore the molecular mechanism of protective drugs.

Methods: In this study, we used 500 μ M H₂O₂ treated RGC-5 cells to induce a cellular oxidative stress model. We treated this cell model with four drug monomers: Propylgallate, Curcumin, Gastrodin and Adenosine to find drug monomers with neuroprotective effect. We used apoptosis PCR array to obtain apoptosis related genes regulated by neuroprotective drugs.

Results: We found the Propylgallate treated RGC-5 cells had highest survival rate when compared to Curcumin, Gastrodin, Adenosine treated RGC-5 cells. In addition, it had lowest cell cytotoxicity and apoptotic rate when compared to Curcumin, Gastrodin, Adenosine treated RGC-5 cells. Moreover, the expression of ROS in Propylgallate treated RGC-5 cells was lowest when compared to Curcumin, Gastrodin, Adenosine treated RGC-5 cells. We found that Caspase-3, Caspase-8, and Caspase-9 are the main target genes of Propylgallate which can preliminarily explain the neuroprotective mechanism of Propylgallate against apoptosis..

Conclusion: The present study revealed that the propylgallate has best neuroprotective effects, it may provide a promising drug to prevent and improve the damage of optic nerve. In this article, we also preliminarily expounded the neuroprotective molecular mechanism of Propylgallate.

Background

Diabetic retinopathy is the most common cause of vision loss among people with diabetes and a leading cause of blindness among working-age adults[1, 2]. Globally, the number of people with DR will row from 126.6 million in 2010 to 191.0 million by 2030, and we estimate that the number with vision-threatening diabetic retinopathy (VTDR) will increase from 37.3 million to 56.3 million^[1]. Recent advances in the field of neuroprotection indicate that healthy neurons can be protected from injury, and damaged neurons can be rescued from dying by blocking specific steps in the cell death cascade[3]. These results suggest that it may be feasible to protect healthy cells and rescue damaged cells in diabetic retinopathy, optic neuropathies, and various retinovascular conditions.

Oxidative stress, which can be defined as an imbalance between the production and removal of reactive oxygen species (ROS), has been implicated in many types of nerve cell death in the central nervous system (CNS) and in the eye[4, 5]. It is well known that oxidative strss is able to induce many types of cell apoptosis via releasing of the reactive oxygen species (ROS) to increase the expression of caspase-3 and caspase-9, including the neurons, cardiomyocytes and nephrocyte[6]. The presence of high concentrations

of ROS can overwhelm the cell's natural defense mechanisms and activate pathways that lead to programmed cell death[5]. There is agreement that certain agents can ameliorate the cell death process in various kinds of injury that involve oxidative stress.

Curcumin is a bright yellow chemical produced by some plants. It is the principal curcuminoid of turmeric (*Curcuma longa*), a member of the ginger family, Zingiberaceae[7]. Gastrodin is a chemical compound which is the glucoside of gastrodigenin. It has been isolated from the orchid *Gastrodia elata* and from the rhizome of *Galeola faberi*[8]. Propyl gallate (PG), a polyphenolic compound family that is synthesized by the condensation of propanol and gallic acid[9]. Adenosine, a nucleoside derived primarily from the extracellular hydrolysis of adenine nucleotides, is a potent regulator of inflammation[10]. These products have been widely used anti-inflammation[11], anti-cancer [12, 13] and other diseases studies[14]. In this study, we explored the anti-oxidative stress ability of these four products. We hypothesized that we can get one product that has best anti-oxidative stress ability. Moreover, we also try to explore its potential mechanism whether the product can protect the retinal ganglion cells against oxidative stress by reducing the expression of apoptotic related gene expression. It will provide a new therapy or prevent the optic nerve injury induced by oxidative stress.

Methods

Materials

Curcumin, Gastrodin, Propylgallate, Adenosine and H_2O_2 were purchased from Sigma (C1386, SBM00313, 48710, A9521, 323381, St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM, Gibco™, 11965092, USA), Fetal bovine serum (FBS, Gibco™, 10099141, USA), penicillin/streptomycin (Gibco™, 15140122, USA) were purchased from Thermo Fisher Scientific. Trypsin was ordered from Invitrogen (Grand Island, 15050065, NY, USA). Cytotoxicity LDH Assay Kit (abam, ab65393, UK) and MTT (abam, ab211091, UK) were purchased from abcam and Apoptosis Detection kit was purchased from Beyotime Institute of Biotechnology (Beyotime, C1062, Nantong, China).

Culture of RGC-5 Cells

RGC-5 cells (ATCC, 2CM3085) were grown in DMEM medium, supplemented with 10% FBS, 1% penicillin/streptomycin. RGC-5 cells were cultured in growth medium and incubated at 37°C in 5% CO_2 .

Cellular oxidative stress model

The oxidative stress model of RGC-5 cells was established by treating RGC-5 cells with 20 μ M, 25 μ M, 100 μ M, 500 μ M H_2O_2 treated for 24 hours.

Proliferation Assay

The effects of curcumin, gastrodin, propylgallate, adenosine on the proliferation of RGC-5 cells under oxidative stress condition were detected by MTT tests. The RGC-5 cells were seeded in 96-well plates at concentration of 2000 cells/ml. The cells were pretreated with different concentration of Curcumin (10, 20, 40 μ M), gastrodin (20, 40, 60 μ M), propylgallate (5, 10, 20 μ M), adenosine (10, 20, 40 μ M) for 2 hours, then treated with 500 μ M H₂O₂ for 24 hours. Cells without any treatment and cells with the treatment of 500 μ M H₂O₂ for 24 hours as control. Following, 10 μ M MTT was added to each well and cultured for 3 hours. Then the medium was removed and 100 μ M DMSO was added to each well. Finally, the plates were read immediately on a plate reader at a test wavelength of 490 nm.

Cytotoxicity Assay

The cytotoxicity of curcumin, gastrodin, propylgallate, adenosine were detected by Cytotoxicity LDH Assay Kit following the manufacturer's instructions. The RGC-5 cells were seeded in 96-well plates at concentration of 2000 cells/ml. The cells were pretreated with different concentration of Curcumin (10, 20, 40 μ M),Gastrodin(20, 40, 60 μ M),Propylgallate(5, 10, 20 μ M), Adenosine(10, 20, 40 μ M) for 2 hours, then treated with 500 μ M H₂O₂ for 24 hours.Following, LDH release agent was added, and the cells were incubated for another 30 minutes, and then 50 μ L of stop solution to each sample well and mix by gentle tapping. Finally, the plates were read immediately on a plate reader at a test wavelength of 490 nm.

Apoptosis assay

The apoptosis levels of the RGC-5 cells treated with different concentration drugs were subsequently analyzed using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit according to the manufacturer's instructions. The cells were seeded in 6-well plates at a density of 1x10⁵ cells/well and pretreated with different concentration of Curcumin (10, 20, 40 μ M),Gastrodin (20, 40, 60 μ M),Propylgallate (5, 10, 20 μ M),Adenosine (10, 20, 40 μ M) for 2 hours, then treated with 500 μ M H₂O₂ for 24 hours.Then cells were then digested with 0.25% trypsin and resuspended in 300 μ l binding buffer containing 5 μ l Annexin V-FITC and 5 μ l propidium iodide solution, and incubated at room temperature in the dark for 20 min. The stained cells were analyzed by flow cytometry (FACScan; BD Biosciences, Franklin Lakes, NJ, USA).

Apoptosis PCR array

An mouse Apoptosis PCR array (PAMM-012A,Qiagen,Frederick, MD., USA) was used to screen a panel of 84 genes representative of apoptosis in RGC-5 cells cell under Propylgallate treated. Total RNA was isolated from RGC-5 cells,RGC-5 cells treated with H₂O₂, RGC-5 cells treated with H₂O₂ + Propylgallate groups using Qiagen RNeasy Mini Kit by following manufacturer's protocol.The first-strand cDNA was mixed with 2 $\times$ RT² SYBR Green qPCR Master Mix and ddH₂O.The qPCR was performed on an Applied Biosystems (ABI) 7500 according to the RT 2 Profiler PCR Array instructions under the following conditions: 95°C for 10 min, then 40 cycles at 95°C for 15 sec and 60°C for 1 min.Microarray data was normalized against the house keeping genes by calculating the Δ Ct for each gene of interestin the plate.Fold changes of gene expression, scatterplot and heatmap were analyzed and generated by using

Western Blot

Whole protein was isolated from RGC-5 cells, RGC-5 cells treated with H₂O₂, RGC-5 cells treated with H₂O₂ + Propylgallate groups using Protein extraction kit (Beyotime, P0028, China) by following manufacturer's protocol. The expression of Caspase-3, Caspase-8 (19677-1-AP, 66093-1-Ig, proteintech) and Caspase-9 (CST, #9508) in RGC-5 cells, RGC-5 cells treated with H₂O₂, RGC-5 cells treated with H₂O₂ + Propylgallate groups were detected by SDS-PAGE.

Statistical analyses

Data are presented as mean $\pm$ standard deviation. SPSS software (v. 20.0, SPSS, Chicago, IL) was used for analysis, and differences among the groups were assessed using one-way ANOVA. *p* values < 0.05 indicated statistical significance. *p* values < 0.01 indicated a significant difference.

Results

Identification of cellular oxidative stress model

In this study, we used H₂O₂ to induce a oxidative stress model of RGC-5 cells. As shown in Fig. 1A, the survival rate of RGC-5 cells treated with 100 μ M and 500 μ M H₂O₂ were significant lower than 20 μ M and 25 μ M H₂O₂ treated RGC-5 cells, especially in 500 μ M H₂O₂ treated RGC-5 cells, the survival rate was about 59.6%. And the cytotoxicity of 100 μ M and 500 μ M H₂O₂ treated RGC-5 cells were about 15.8% and 20.1% respectively, which was obvious higher than the other 3 groups (Fig. 1B). In addition, the apoptosis assay results discovered that the apoptotic rate of 100 μ M and 500 μ M H₂O₂ treated RGC-5 cells were 5.47% and 8.53% respectively, which was obvious higher than the other 3 groups (Fig. 1C). Finally, the ROS expression in 100 μ M and 500 μ M H₂O₂ treated RGC-5 cells were 51.11% and 62.96% respectively, which was obvious higher than the other 3 groups (Fig. 1D). According to the above results, we used 500 μ M H₂O₂ to induce oxidative stress model of RGC-5 cells for this study.

The protective effects of Curcumin, Gastrodin, Propylgallate, Adenosine on the proliferation of RGC-5 cells under oxidative stress condition

Firstly, we examined the effects of Curcumin, Gastrodin, Propylgallate, Adenosine on the proliferation of RGC-5 cells under oxidative stress condition. As Shown in Fig. 2A, the survival rate of 500 μ M H₂O₂ treated RGC-5 cells was about 67.1%. The survival rate of 10 and 20 μ M curcumin treated RGC-5 cells was about 72.2% and 72.2% respectively, which was higher than the control. By comparison, the 40 μ M of Curcumin was able to significantly reduce the survival rate of 500 μ M H₂O₂ pretreated RGC-5 cells, which was only

about 40.5%.The survival rate of 20, 40, 60μM Gastrodin treated RGC-5 cells was 65.7%, 67.7% and 68.7%, which was no significant difference with the control group (Fig. 2B). Interestingly, the survival rate of 5, 10, 20 μM Propylgallate treated RGC-5 cells was 92.3%, 87.7% and 87.9%, which was significant higher than the control group (Fig. 2C).Finally, the survival rate of 10, 20, 40μM Adenosine treated RGC-5 cells was 72.3%, 74.6% and 72.9%, which was higher than the control group (Fig. 2D).

The cytotoxicity of Curcumin,Gastrodin,Propylgallate,Adenosine pretreated RGC-5 cells under oxidative stress condition

As Shown in Fig. 3A, the cytotoxicity of 500μM H₂O₂ treated RGC-5 cells was about 44.8%. The cytotoxicity of 10, 20, 40μM Curcumin treated RGC-5 cells was 44.3%, 44.1% and 48%, which was no significant difference with the control group (Fig. 3A).The cytotoxicity of 20, 40, 60μM Gastrodin treated RGC-5 cells was 44.7%, 45.6% and 44.6%, which was no significant difference with the control group (Fig. 3B).Interestingly, the cytotoxicity of 5, 10, 20μM Propylgallate treated RGC-5 cells was 40.6%, 41.4% and 40.4%,which was significant lower than the control group (Fig. 3C).Finally, the cytotoxicity of 10, 20, 40μM Adenosine treated RGC-5 cells was 45.2%, 45.3% and 46.1%, which was no significant difference with the control group (Fig. 3D).

The apoptotic rate of Curcumin,Gastrodin,Propylgallate,Adenosine pretreated RGC-5 cells under oxidative stress condition

As Shown in Fig. 4A, the apoptotic rate of 500μM H₂O₂ treated RGC-5 cells was about 13.47%. The apoptotic rate of 10, 20μM curcumin treated RGC-5 cells was 10.62% and 12.05%.which was no significant difference with the control group. While the apoptotic rate of 40μM Curcumin treated RGC-5 cells was 41.08%, it was significant higher than the control group (Fig. 4A). The apoptotic rate of 20, 40, 60μM Gastrodin treated RGC-5 cells was 13.73%, 13.7% and 11.53%, which was no significant difference with the control group (Fig. 4B).Interestingly, the apoptotic rate of 5, 10, 20 μM Propylgallate treated RGC-5 cells was 8.43%, 9.11% and 9.29%, which was significant lower than the control group (Fig. 4C). Finally, the Apoptotic rate of 10, 20, 40 μM adenosine treated RGC-5 cells was 10.53%, 11.83% and 14.18%, which was no significant difference with the control group (Fig. 4D).

The ROS expression of Curcumin,Gastrodin,Propylgallate,Adenosine pretreated RGC-5 cells under oxidative stress condition

As shown in Fig. 5A,the expression of ROS in control group was about 29.30%.The expression of ROS in 10, 20, 40μM Curcumin treated RGC-5 cells was 62.91%, 88.92% and 98.79% respectively, which was

significant higher than the control group. The expression of ROS in 20, 40, 60 μ M Gastrodin treated RGC-5 cells was 26.26%, 27.61% and 26.17%, which was no significant difference with the control group (Fig. 5B). Interestingly, the ROS expression of 5, 10, 20 μ M Propylgallate treated RGC-5 cells was 9.6%, 11.11% and 12.59%, which was significant lower than the control group (Fig. 5C). The ROS expression of 10, 20, 40 μ M Adenosine treated RGC-5 cells was 55.71%, 47.01% and 53.94%, which was significant higher than the control group (Fig. 5D).

The apoptosis related genes expression of RGC-5 cells treated with Propylgallate under oxidative stress condition

Previous experimental results showed that Propylgallate had a significant anti-apoptotic effect, in order to obtain the anti-apoptotic molecular mechanism of Propylgallate, we used the Apoptosis PCR array to obtain the apoptosis related genes regulated by Propylgallate. According to the results of the Apoptosis PCR array (Fig. 6A), the RNA expression of Caspase-3, Caspase-8, and Caspase-9 is in line with the trend of propylgallate (other genes data not shown). As shown in Fig. 6B and C, the RNA and protein expression of Caspase-3, Caspase-8, and Caspase-9 in RGC-5 cells treated with H_2O_2 were significantly higher than the control of RGC-5 cells. Suggesting the H_2O_2 treatment increased RGC-5 cell apoptosis. Interestingly, the expression of Caspase-3, Caspase-8, and Caspase-9 in RGC-5 cells treated with propylgallate after H_2O_2 pretreatment was obviously reduced. These results suggesting propylgallate was able to protect RGC-5 cells against the injury of oxidative stress.

Discussion

Diabetic retinopathy is a group of eye conditions that damage the optic nerve, the health of which is vital for good vision. It is one of the leading causes of blindness for people over the age of 60. It can occur at any age but is more common in older adults. Bilateral blindness will be present in 8.4 million people in 2010, rising to 11.2 million people in 2020, respectively [15]. It has seriously affected the people's health and burdened the government healthcare system. In this study, we compared the neuroprotective effect of curcumin, gastrodin, propylgallate, adenosine, and we found the propylgallate has the best neuroprotective effect.

In this study, we used H_2O_2 to induce cellular oxidative stress model in RGC-5 cells, and we found 500 μ M H_2O_2 could successfully establish cellular oxidative stress model. The 500 μ M H_2O_2 treated RGC-5 cells had the lowest survival rate (59.6%) and highest LDH release percentage (26.0%), apoptotic rate (8.53%) and ROS expression (62.96%). So we used 500 μ M H_2O_2 to induce oxidative stress in RGC-5 cells for this study.

Curcumin [16], Gastrodin [17], Propylgallate [18], Adenosine [19] were reported that they were able to against the H_2O_2 induced oxidative stress in other cells. However, there are few reports revealed that Curcumin, Gastrodin, Propylgallate, Adenosine were able to relieve the damage induced by oxidative stress in RGC-5 cells. In this study, we found the Propylgallate treated RGC-5 cells had the highest survival rate when

compared to Curcumin, Gastrodin, Adenosine treated RGC-5 cells. In addition, it had lowest cell cytotoxicity and apoptotic rate when compared to Curcumin, Gastrodin, Adenosine treated RGC-5 cells. Moreover, the expression of ROS in propylgallate treated RGC-5 cells was lowest when compared to Curcumin, Gastrodin, Adenosine treated RGC-5 cells. Furthermore, we also demonstrated that Propylgallate could inhibit the expression of Caspase-3, Caspase-8, Caspase-9 in H₂O₂ pretreated RGC-5 cells in protein level, which is more convincing. According to the aforementioned results, the present study revealed that the propylgallate has best neuroprotective effects, it may provide a promising drug to prevent and improve the damage of optic nerve.

Declarations

ACKNOWLEDGEMENT

This study was supported by grants from the Natural Science Foundation of Inner Mongolia Autonomous Region of China (2017MSLH0822) and (2018MS08119).

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Identification of cellular oxidative stress model

Figure 2

The protective effects of Curcumin, Gastrodin, Propylgallate, Adenosine on the proliferation of RGC-5 cells under oxidative stress condition

Figure 3

The cytotoxicity of Curcumin, Gastrodin, Propylgallate, Adenosine pretreated RGC-5 cells under oxidative stress condition

Figure 4

The apoptotic rate of Curcumin, Gastrodin, Propylgallate, Adenosine pretreated RGC-5 cells under oxidative stress condition

Figure 5

The ROS expression of Curcumin, Gastrodin, Propylgallate, Adenosine pretreated RGC-5 cells under oxidative stress condition

Figure 6

The expression of Caspase-3, Caspase-8, Caspase-9 of RGC-5 cells treated with H2O2, H2O2+ Propylgallate, the RGC-5 cells without any treatment as control.