Puerarin protects the retinal pigment epithelium (ARPE19) against hypoxia-induced blood-retinal barrier breakdown and Akt/Bcl2-dependent apoptosis

Minh-Anh Nguyen Ngo Le

Ho Chi Minh City University of Medicine and Pharmacy

Yao-Tseng Wen

Buddhist Tzu Chi General Hospital Hualien: Hualien Tzu Chi Hospital

Dieu-Thuong Thi Trinh

Ho Chi Minh City University of Medicine and Pharmacy

Rong - Kung Tsai (✉ rksai@tzuchi.com.tw)

Institute of Eye Research, Buddhist Tzu Chi General Hospital, Tzu Chi University <https://orcid.org/0000-0001-8760-5739>

Research

Keywords: Puerarin, Human retinal pigment epithelium cells, Hypoxia, Apoptosis, PI3K/Akt pathway

Posted Date: March 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-249980/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Puerarin, an isoflavonoid, is a neuroprotectant against many ischemic brain injuries. The research purpose was to investigate whether puerarin treatment can inhibit hypoxia-induced apoptosis and barrier breakdown on human retinal pigment epithelium (ARPE-19) cells.

Methods: Treatment with 100 μM of puerarin showed no ARPE-19 cytotoxicity. Treatments with 50 μM or 100 μM of puerarin significantly preserved cell viability under hypoxic conditions for 12 h ($p < 0.05$). Moreover, treatments with 50 μM and 100 μM of puerarin significantly reduced the proportion of Annexin V- and PI-positive ARPE cells under hypoxic conditions ($p < 0.05$). The TER analysis and junctional protein staining demonstrated that treatments with 50 μM or 100 μM of puerarin prevented the hypoxia-induced barrier disruption in ARPE-19 cultures. In contrast to the untreated group, treatment with puerarin significantly increased the level of Bcl-2 and decreased the levels of p-Bad, Bax, and cleaved caspase 3 in RPE cell under hypoxic conditions ($p < 0.05$). Treatment with puerarin maintained Akt1 activation in the ARPE-19 cells under hypoxic conditions ($p < 0.05$), and inhibition of PR-induced Akt1 activation abolished the protective effect of puerarin in ARPE-19 cells under hypoxic conditions.

Conclusion: Our results demonstrated that treatment with puerarin protected RPE cell against hypoxia-induced blood-retinal barrier breakdown and Akt-dependent apoptosis. These findings suggest that puerarin could be developed as an alternative treatment for ischemic and hypoxic retinal injuries.

Background

The primary cause of blindness among people older than 50 years is reported to be age-related macular degeneration (AMD). Advanced dry AMD is characterized by photoreceptor and retinal pigment epithelium (RPE) loss. (1) Studies have suggested that drusen between the RPE layer and the choriocapillaries may block the oxygen diffusion and nutrients supply from the choriocapillaries to the RPE layer and photoreceptors. (2, 3) Lack of sufficient blood supply to the retina results in hypoxia, tissue ischemia, and the fast defeat of energy generation with subsequent processes of cell death. (4) RPE and photoreceptor cells die gradually under retinal ischemia; therefore, hypoxia and ischemia have been associated with the pathophysiology of retinal degeneration. (4) In wet AMD, hypoxia may induce expression of angiogenic factors, producing choroid neovascularization. (5) In addition, hypoxia is a detrimental factor for the blood–retina barrier (BRB) and leads to the progression of wet AMD. (6) Protecting RPE cells from hypoxia is crucial to prevent or reduce the progression of retinal degenerations.

Puerarin is the primary bioactive ingredient from the *Pueraria lobata* root, widely known as “Gegen” in traditional Chinese medicine. It possesses a broad spectrum of pharmacological effects such as vasodilation,(7) cardio-protection,(8) neuroprotection,(9) and antioxidant ,(10) anticancerous, (11) and anti-inflammation properties; (12) in addition to inhibiting diabetic ocular complications. (13) Studies have shown that puerarin has neuroprotective effects against toxicities induced by glutamate,(14) N-methyl-D-aspartic acid,(15) β -amyloid, (16) and 1-methyl-4- phenylpyridinium (MPP+).(17) In vivo have

also demonstrated that puerarin is neuro-protectant in rat models of spinal cord (18, 19) and cerebral ischemia injuries (20, 21) through activation of the PI3k/Akt signaling pathway. (9) Thus, puerarin may be an ideal protective candidate for RPE cells against hypoxic stress.

RPE is the pigmented cell layer in the inner sensorineural layer on the Bruch's membrane. (22) The RPE transports oxygen and nutrients from the choroid to the photoreceptor cells, while also remove wastes of photoreceptors for disposal to the choroid. However, an insufficient supply of nutrients and oxygen from choroidal capillaries result in RPE damage. (23) Remarkably, Akt activation was reported to protect RPE cells under normal conditions and during disease circumstances such as AMD. (24) Moreover, puerarin has been reported to activate the Akt pathway to produce its antiapoptosis effects in neuroblastoma, (17) cardiomyocyte, (25) and pancreatic β cells. (26) Thus, we hypothesized that puerarin might trigger the Akt activation to prevent RPE cell death under hypoxic conditions. We evaluated the possible cytoprotective effects of puerarin in hypoxia-induced RPE cell apoptosis and its possible mechanisms.

Methods

1.1 Culture of ARPE-19

The human retinal pigment epithelial cell line, ARPE-19, was expanded in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Grand Island, NY, USA) containing 3 mM L-glutamine (Gibco), 0.5% fetal bovine serum (Gibco), 100 U/mL penicillin G, and 100 g/mL streptomycin sulfate (Gibco) at 37°C in an environment containing 5% CO₂. Cell suspensions were added into 96-well microplates at 5000 cells/well in 100 μ L. The ARPE-19 cells were incubated overnight to reach 80% to 90% confluence before further experiments.

1.2 Hypoxic conditions

RPE cells were added into 96-well plates at a density of 5×10^3 cells in each well. After 24h, the culture was washed with phosphate buffered saline (PBS), before adding fresh culture medium to them. For the hypoxia experiment, cells were incubated at 37°C in a controlled chamber at 1% O₂ in the presence of 5% CO₂ and 94% N₂ (ASTEC, Fukuoka, Japan). Cells were incubated under normal conditions (20% O₂, 5% CO₂, and 75% N₂) served as normoxic controls.

1.3 Cell viability assay

We measured cell viability using WST-8 cell proliferation assay kits (WST-8, Cayman, USA) in triplicate for each group according to the manufacturer's guideline. We read the OD_{450 nm} in each well to assess cell viability.

1.4 Detection of apoptosis

We detected apoptosis in ARPE-19 by using the Annexin V PI Apoptosis Detection Kit (Genedirex, USA). Briefly, ARPE-19 cells were reacted with Annexin V (1 mg/ml, Genedirex) and propidium iodide (PI) solution (5 mg/ml, Genedirex) for 20 min. An epi-fluorescence microscope (Axioskop; Carl Zeiss Meditech, Thornwood, NY, USA) was used to detect early (Annexin V⁺/PI⁻) and late (Annexin V⁺/PI⁺) apoptotic cells. We calculated 6 randomly selected areas of 62,500 μm^2 in each well and calculated their averages as the mean density of apoptotic cells. All detections were performed in triplicate.

1.5 Measurement of transepithelial electrical resistance

To ensure the development of a properly formed monolayer and tight junctions within the Transwell plates, we measured the transepithelial electrical resistance (TER) using an epithelial volt-ohm meter (World Precision Instruments, Sarasota, FL, USA), after applying the designed treatments to the cultures. To determine TER (Ω/cm^2), we subtracted the values of Transwells without cells from the experimentally measured values.

1.6 Immunofluorescence staining of tight junction proteins

We immunostained the ARPE-19 cell monolayer on glass coverslips for tight junction proteins, ZO-1 and Occludin. Briefly, cells were fixed in methanol for 15 min and blocked by 2% bovine serum albumin buffer (Sigma-Aldrich Corp, USA) for 1 hour. Rabbit anti-Occludin and mouse anti-ZO-1 (1: 200 dilution; Abcam, Cambridge, MA, USA) were added and incubated overnight. The next day Alexa 488 secondary antibodies (1:200 dilution; Invitrogen; Thermo Fisher, USA) were added for 1 hour at 25 °C. Pictures were taken by using a fluorescence microscope (Axioskop; Carl Zeiss Meditech, Thornwood, NY, USA).

1.7 Western blot analysis

We collected cells after 24 hours under hypoxic conditions in each group. We used Western blots to quantitate levels of pro-apoptosis and antiapoptosis proteins, tight junction proteins, and PI3/Akt phosphorylation, ERK phosphorylation in each group. The 4-12% NuPAGE was used to separate the protein extracts. The gels were transferred onto polyvinylidene difluoride membranes. The membranes were then reacted with cleaved caspase-3, p-Bad, Bax, Bcl-2, p-Akt, p-PI3K, p-ERK, ZO-1, and Occludin antibodies (Cell signaling, USA) primary antibodies at 4 °C. Next, the membranes were reacted with a secondary antibody for 1 hour. Signals were developed by using an enhanced chemiluminescent substrate, and images were acquired by a Western blot analyzer. We calculated the relative density using the ImageJ software (<https://imagej.nih.gov/ij/>). In brief, the mean gray value was measured to quantify the protein band in the quantification software. For band detection, the rectangle tool was used to select a region of interest. For band quantification, the area under the curve of the detected band was used to represent the band density. The final relative density is the ratio of protein of interest to internal loading control.

1.8 Statistical analysis

All statistical analyses were performed using a GraphPad Prism. Data are presented as means $\pm$ standard deviations. We applied Mann–Whitney U-tests for comparisons between groups and considered $P < 0.05$ as statistically significant.

Results

2.1 Cytotoxicity of puerarin in the ARPE-19 cell

We examined puerarin cytotoxicity by incubating ARPE-19 cells with the designed-concentrations of puerarin (10, 100, 200, 500, and 1000 μM) for 24 hours, and using WST-8 assays to determine their viability. The viability of RPE cells was significantly reduced by treatment with 500 and 1000 μM puerarin compared to the viability of untreated-RPE cells (Figure 1A; 500 μM puerarin, $P = 0.029$; 1000 μM puerarin, $P = 0.0006$). To measure long-term puerarin effects on cytotoxicity, we treated the ARPE-19 cells with puerarin at 10, 100, and 200 μM for 1, 2, 5, and 7 days. The ARPE-19 cells treated with 200 μM of puerarin showed lower viability at 2, 5, and 7 days posttreatment (Figure 1B, $p < 0.0001$). Puerarin concentrations lower than 100 μM resulted in no cytotoxicity in the ARPE-19 cells during 7 days in culture.

2.2 Puerarin reduced the hypoxia-induced apoptosis in ARPE-19 cells

RPE cell death progressed in a time-dependent manner under hypoxic culture conditions (Figure 2A). We observed a dramatic decrease in ARPE-19 cell viability 8 hours after initiating the incubation in the anaerobic chamber (Figure 2A, $P = 0.0001$). Puerarin at 50 and 100 μM inhibited the hypoxia-induced RPE cell death significantly. However, puerarin at 10 μM had no significantly protective effect on ARPE-19 cells after the same hypoxic incubation period (Figure 2A, $P = 0.0001$). Next, we examined whether the protective effect is involved in an antiapoptotic response. The Annexin V staining demonstrated that puerarin treatment at 50 and 100 μM reduced the proportion of Annexin V-positive and PI-positive cells under hypoxic conditions (Figure 2B). The proportions of Annexin V-positive cells in the hypoxic control, the 10 μM PR-treated, the 50 μM PR-treated, and the 100 μM PR-treated cells were $55.3 \pm 5.5\%$, $50.7 \pm 7.0\%$, $14.6 \pm 4.5\%$, and $12.8 \pm 3.9\%$, respectively. The proportions of PI-positive cells in the hypoxic control, the 10- μM PR-treated, the 50- μM PR-treated, and the 100- μM PR-treated cells were $72.5 \pm 6.6\%$, $63.7 \pm 4.0\%$, $21.7 \pm 9.4\%$, and $21.0 \pm 5.9\%$, respectively (Figure 2B). Treatment with 50 μM puerarin reduced the percentage of Annexin V-positive cells 40.7% ($P = 0.0004$) and that of PI-positive cells 50.8% ($P = 0.0004$) compared to the percentages in the hypoxic control group. Treatment with 100 μM puerarin reduced the percentage of Annexin V-positive cells 42.5% ($P = 0.0003$) and that of PI-positive cells 51.5% ($P = 0.0002$) compared to the percentages in the hypoxia control cells. These results indicate that treatments with 50 or 100 μM of puerarin provided antiapoptotic capabilities in ARPE-19 cells against hypoxic conditions.

2.3 Puerarin inhibited the hypoxia-induced barrier disruption in ARPE-19 cells

The TER of ARPE-19 cell was measured under normoxic and hypoxic conditions and after treatment with or without puerarin (50 μM or 100 μM). The TERs of ARPE-19 cells under normoxic, hypoxic without puerarin, hypoxic with puerarin (50 μM), and hypoxic with puerarin (100 μM) conditions were 161.1 ± 9.6

$\Omega \text{ cm}^2$, $66.1 \pm 3.5 \Omega \text{ cm}^2$, $127.1 \pm 7.2 \Omega \text{ cm}^2$, and $126.4 \pm 7.3 \Omega \text{ cm}^2$, respectively. Treatment with 50 and 100 μM of puerarin significantly inhibited the hypoxia-induced TER decreases by 1.92-fold ($p < 0.0001$) and by 1.91-fold ($p < 0.0001$), respectively, compared to the TER decrease in the hypoxia without puerarin group (Figure 3A).

To confirm the TER results, we quantitated the tight junctional proteins ZO-1 and Occludin by immunocytochemistry and Western blotting analysis. The expression of levels of ZO-1 and Occludin were significantly reduced 2.5-fold ($P = 0.0186$) and 1.86-fold ($P = 0.0002$) in ARPE-19 cells under hypoxia conditions compared to those under normoxic conditions. Treatment with 50 μM of puerarin significantly increased the protein levels of ZO-1 1.99-fold ($p = 0.0148$) and those of Occludin 1.45-fold ($p = 0.0116$) in ARPE-19 cells under hypoxic conditions compared to the levels in cells without puerarin treatment. Treatment with 100 μM puerarin in ARPE-19 cells under hypoxia also significantly increased the levels of ZO-1 and Occludin 1.99-fold ($p = 0.018$) and 1.41-fold ($p = 0.02$), respectively, compared to the levels in cells without puerarin treatment (Figure 3B).

Treatment with 50 μM and 100 μM puerarin prevented the disorganization of ZO-1 and Occludin induced by hypoxia (Figure 3C). These results indicate that puerarin inhibits hypoxia-induced disruption of the barrier function in ARPE-19 cells.

2.4 Puerarin reduced the levels of apoptosis-associated factors

To elucidate the potential mechanism by puerarin attenuating hypoxia-induced apoptosis, we performed a Western blot analysis to determine a set of apoptosis-associated factors, including cleaved caspase 3, p-Bad, Bax, and Bcl-2. We incubated ARPE-19 cells with 50 and 100 μM puerarin and exposed them to hypoxia for 24 hours before quantitating the proteins.

The levels of p-Bad, Bax, and cleaved caspase 3 were increased by 18-fold ($P < 0.0001$), 1.37-fold ($P = 0.0368$), and 15.24-fold ($P < 0.0001$), respectively, in the hypoxia groups compared with the levels in the normoxia group (Figure 4). The level of Bcl-2 was decreased by 2.24-fold ($P = 0.0004$) in the hypoxia group compared with that in the normoxia group (Figure 4). Puerarin (50 μM) treatment reduced the levels of p-Bad, Bax, and cleaved caspase 3 by 1.57-fold ($P = 0.0022$), 1.36-fold ($P = 0.0432$), and 1.81-fold ($P = 0.0003$), respectively, compared with the levels without puerarin treatment under hypoxic conditions. Puerarin (100 μM) treatment reduced the levels of p-Bad, Bax, and cleaved caspase 3 by 2.05-fold ($P = 0.0002$), 1.43-fold ($P = 0.0214$), and 1.96-fold ($P = 0.0002$), respectively, compared with the levels without puerarin treatment under hypoxic conditions. The level of Bcl-2 was increased 2.1-fold ($P = 0.0009$) in the cells treated with puerarin (50 μM) and 2.16-fold ($P = 0.0006$) in the cells treated with puerarin (100 μM) than in the cells under hypoxia without puerarin (Figure 4). These observations imply that puerarin induced the level of Bcl-2, reduced the levels of Bax and Bad, cleavage of caspase 3, and exhibited antiapoptotic properties through the mitochondrial apoptotic pathway.

2.5 Puerarin induced Akt activation and reduced Akt1-dependent apoptosis

PI3K/Akt (27) and MAPK/ERK (28) pathways regulate cell proliferation and survival. In this study, we examined the pathways of PI3K/Akt and MAPK/ERK. The ARPE-19 cells were exposed to hypoxic conditions for 24 h. Our results showed that hypoxic conditions significantly induced the expression of p-Akt1 1.46-fold ($P = 0.0019$) and that of p-ERK 1.235-fold ($P = 0.023$) with a maximal effect after 3 hours compared with expression levels under normoxic conditions. However, the level of p-Akt1 significantly decreased after 12 hours of exposure to hypoxia compared to normoxia (Figure 5A, $P < 0.0001$). The level of pERK showed no statistical difference among each group (Figure 5A).

In order to elucidate whether the Akt inhibitor LY 294002 had an effect on PR-induced PI3K/Akt1 activation, we incubated the ARPE-19 cells with puerarin plus LY 294002 (20 μ M) under hypoxic conditions for 24 hours. Our results revealed that puerarin treatment increased the phosphorylation of PI3K 1.66-fold ($P = 0.0015$), and that of p-Akt1 7.99-fold ($P < 0.0001$) under hypoxic conditions, compared with the phosphorylation in cells without treatment under hypoxic conditions. However, inhibition of p-Akt by LY 294002 20 μ M abrogated this effect (Figure 7B, $P = 0.365$).

Puerarin treatment activated the PI3K/Akt signaling pathway in ARPE-19 cells under hypoxic condition, and activation of the PI3K/Akt pathway is crucial for the regulation of apoptosis. We further examined the PI3K/Akt1-dependent apoptosis for puerarin treatment. Figure 5D shows the results of Annexin V-FITC/PI stainings to detect apoptotic cells. The percentages of Annexin V-positive cells were $50.87 \pm 6.64\%$, $18.33 \pm 3.81\%$, and $54.33 \pm 7.28\%$, and those of PI-positive cells were $56.12 \pm 4.01\%$, $18.33 \pm 6.29\%$, and $53.41 \pm 4.5\%$, respectively in cells without puerarin treatment, cells treated with puerarin (50 μ M), and cells treated with puerarin (50 μ M) combined with LY294002 (20 μ M). We found no significant differences between cells treated with puerarin (50 μ M) combined with LY294002 (20 μ M) and those without treatments under hypoxic conditions ($p = 0.8819$ for Annexin V-positive cells and $p = 0.7762$ for PI-positive cells). The difference between cells treated with puerarin (50 μ M) and those treated with puerarin (50 μ M) combined with LY294002 (20 μ M) was statistically significant ($p = 0.0011$ for Annexin V-positive cells and $p < 0.0001$ for PI-positive cells). According to these results, puerarin treatment reduced the hypoxia-induced RPE apoptosis by activating Akt signaling pathway. Besides, inhibiting the Akt activation by LY294002 removed the protective effects of puerarin against apoptosis in hypoxia-induced ARPE-19 cells, suggesting the puerarin antiapoptotic properties on hypoxic RPE cells are dependent on PI3K/Akt signaling.

Discussion

Hypoxia-ischemia underlies various blinding ocular conditions such as diabetic retinopathy, AMD, and retinal detachment. In addition, these problems are collectively referred to as ischemic retinopathies.(29) The ischemic retinal injury occurs through a self-reinforcing damaging mechanism involving calcium influx, oxidative stresses resulted from hypoxia and energy failure, decrease in mitochondrial enzyme activity, and increase glutamatergic stimulation.(30) A strategy against hypoxia-induced retinal cell damage could restore the healthy environment of the retina and avoid retinal injury. Our results demonstrated that puerarin protected RPE cells against hypoxia-induced cell damages. We found that

puerarin treatment regulated Bcl-2/Bax/caspase-3 signaling pathway to prevent hypoxia-induced apoptosis in ARPE-19 cells. In addition, puerarin maintained Akt1 activation to keep survival of ARPE-19 cell under hypoxic condition. Moreover, Puerarin reduced hypoxia-induced blood-retinal barrier breakdown. Thus, our findings suggested that puerarin may be a potential drug for AMD and ischemic retinopathy.

Puerarin has shown strong neuroprotective effects during ischemia/reperfusion, protecting neurons by activating antiapoptotic mechanisms .(20, 31, 32) Thus, we believe puerarin may be a promising candidate to treat retinal degenerative diseases. However, there is no study to investigate the role of puerarin in hypoxia induced apoptotic damage on RPE cells. In this study, we demonstrated that puerarin played a protective role against hypoxia-induced ARPE-19 apoptosis via the Akt1 activation and modulation of Bcl-2/Bax/caspase-3 signaling pathway. These findings suggest that puerarin displayed the antiapoptotic capability through the mitochondrial apoptotic pathway. Mitochondria is a critical element in regulating apoptosis. Antiapoptotic Bcl-2-associated proteins suppressed the activity of Bax. (33) Apoptosis was reported to be suppressed by puerarin treatment in various cell types via regulation of the Bcl-2 family proteins .(9, 34) Taken together, we suggested that puerarin provide the anti-apoptotic activity by modulating Bcl-2, Bax, and Bad in ARPE-19 cells under hypoxic condition.

The inner BRB directly regulates the flux into the inner retina to maintain the homeostasis of the retina. (23, 35) The well-developed tight junctions are crucial for the regulation of fluid and solutes that cross the BRB. In this study, treatment with puerarin kept the TER value in ARPE-19 cells under hypoxic condition. This effect was involved in the regulation of tight junction proteins. Many proteins were reported to be associated with tight junctions. Among them, Occludin and ZO-1 are the well-studied in many cells.(36) In the present study, we found that hypoxia exposure decreased the protein levels of ZO-1 and Occludin in ARPE-19 cells. Hypoxia-induced disorganization of ZO-1 and Occludin were preserved by puerarin treatment. Interestingly, we also found that treatment with puerarin induced Akt1 activation in the ARPE-19 cells under hypoxic condition. In addition, the Akt1 activation prevented the RPE disruption in the hypoxic condition. The previous report demonstrated that erythropoietin-induced Akt activation preserved RPE barrier function in the APRE-19 cells to prevent RPE disruption.(37) Thus, we considered that puerarin-induced Akt1 activation might be involved in RPE barrier function to maintain the organization of junctional proteins in the ARPE-19 cells.

The PI3K/Akt pathway can regulate cell proliferation, differentiation, and survival. This pathway is activated by some growth factors and specific extracellular signals.(27) The PI3K/Akt pathway was reported that its activation is involved in the protection of ARPE-19 cells from apoptosis.(38–40) Our data demonstrated that puerarin triggered Akt1-dependent cell survival in APRE-19 cells in response to hypoxic stress. The Akt1 activation can regulate cell-survival signals through the Bcl-2 family.(41) Therefore, we suggested that puerarin regulated Akt1/Bcl-2 pathway in response to hypoxia for RPE survival.

In summary, our results suggest that treatment with puerarin prevents the hypoxia-induced apoptosis in ARPE-19 cells via modulation of Akt/Bcl2 pathway. In addition, treatment with puerarin inhibited the

hypoxia-induced disruption of BRB. These results indicate that puerarin may be developed as an alternative treatment for ischemic and hypoxic retinal injuries.

Abbreviations

AMD

age-related macular degeneration

PR

Puerarin

RPE

retinal pigment epithelium

PBS

phosphate buffered saline

TER

transepithelial electrical resistance

BRB

blood retinal barrier

Bcl-2

B-cell lymphoma 2

ZO-1

Zonula occludens-1

Bax

Bcl-2 associated X-protein

Declarations

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of supporting data

Raw data can be obtained from corresponding authors.

Competing interests

The authors declare no competing interests

Funding

This study was supported by a Ministry of Science and Technology Grant from the Taiwanese Government (MOST 103-2314-B-303-007-MY3).

Author contributions

Y-T W, M-A-N-N-L and R-K T designed the experiments. M-A-N-N-L performed the experiment. M-A-N-N-L, Y-T W and D-T-T-T analyzed the data. R-K T contributed the reagents/materials/ analysis tools. M-A-N-N-L, Y-T W and R-K Tsai wrote the manuscript. All contributing authors have read and approved the final version of the manuscript.

Acknowledgments

The authors thank I-Ping Tsai and Yi-Ling Zhang for their assistance with experimental handling and image preparation. This manuscript was edited by Wallace Academic Editing. This study was supported by a Ministry of Science and Technology Grant from the Taiwanese Government (MOST 103-2314-B-303-007-MY3).

Author information

Affiliations:

Institute of Eye Research, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan.

Yao-Tseng Wen, Minh-Anh Nguyen Ngo Le, and Rong-Kung Tsai

Institute of Medical Sciences, Tzu Chi University, Hualien, Taiwan.

Rong-Kung Tsai

Faculty of Traditional Medicine, Univeristy of Medicine and Pharmacy at Ho Chi Minh City, Viet Nam.

Minh-Anh Nguyen Ngo Le, Dieu-Thuong Thi Trinh

Corresponding author:

Correspondence to Rong-Kung Tsai

References

1. Hanus J, Anderson C, Sarraf D, Ma J, Wang S. Retinal pigment epithelial cell necroptosis in response to sodium iodate. *Cell Death Discov.* 22016. p. 16054-.
2. van Kuijk FJ, McPherson SW, Roehrich H. Enhanced Detection of Sub-Retinal Pigment Epithelial Cell Layer Deposits in Human and Murine Tissue: Imaging Zinc as a Biomarker for Age-Related Macular

- Degeneration (An American Ophthalmological Society Thesis). Transactions of the American Ophthalmological Society. 1152017.
3. Zhao R, Camino A, Wang J, Hagag AM, Lu Y, Bailey ST, et al. Automated drusen detection in dry age-related macular degeneration by multiple-depth, en face optical coherence tomography. *Biomed Opt Express*. 82017. p. 5049-64.
 4. Kaur C, Foulds WS, Ling EA. Hypoxia-ischemia and retinal ganglion cell damage. *Clin Ophthalmol*. 22008. p. 879 – 89.
 5. Alizadeh E, Mammadzada P, André H. The Different Facades of Retinal and Choroidal Endothelial Cells in Response to Hypoxia. *International journal of molecular sciences*. 192018.
 6. Kaur C, Foulds WS, Ling EA. Blood-retinal barrier in hypoxic ischaemic conditions: basic concepts, clinical features and management. *Prog Retin Eye Res*. 2008;27(6):622–47.
 7. Deng Y, Ng ES, Yeung JH, Kwan YW, Lau CB, Koon JC, et al. Mechanisms of the cerebral vasodilator actions of isoflavonoids of Gegen on rat isolated basilar artery. *J Ethnopharmacol*. 2012;139(1):294–304.
 8. Zhang SY, Chen G, Wei PF, Huang XS, Dai Y, Shen YJ, et al. The effect of puerarin on serum nitric oxide concentration and myocardial eNOS expression in rats with myocardial infarction. *J Asian Nat Prod Res*. 2008;10(3–4):373–81.
 9. Han JQ, Yu KY, He M. [Effects of puerarin on the neurocyte apoptosis and p-Akt (Ser473) expressions in rats with cerebral ischemia/reperfusion injury]. *Zhongguo Zhong xi yi jie he za zhi Zhongguo Zhongxiyi jiehe zazhi = Chinese. journal of integrated traditional Western medicine*. 2012;32(8):1069–72.
 10. Liu CM, Ma JQ, Sun YZ. Puerarin protects rat kidney from lead-induced apoptosis by modulating the PI3K/Akt/eNOS pathway. *Toxicol Appl Pharmacol*. 2012;258(3):330–42.
 11. Lin YJ, Hou YC, Lin CH, Hsu YA, Sheu JJ, Lai CH, et al. Puerariae radix isoflavones and their metabolites inhibit growth and induce apoptosis in breast cancer cells. *Biochem Biophys Res Commun*. 2009;378(4):683–8.
 12. Hu W, Yang X, Zhe C, Zhang Q, Sun L, Cao K. Puerarin inhibits iNOS, COX-2 and CRP expression via suppression of NF-kappaB activation in LPS-induced RAW264.7 macrophage cells. *Pharmacol Rep*. 2011;63(3):781–9.
 13. Hao LN, Wang M, Ma JL, Yang T. Puerarin decreases apoptosis of retinal pigment epithelial cells in diabetic rats by reducing peroxynitrite level and iNOS expression. *Sheng Li Xue Bao*. 2012;64(2):199–206.
 14. Wang K, Zhu X, Zhang K, Wu Z, Sun S, Zhou F, et al. Neuroprotective Effect of Puerarin on Glutamate-Induced Cytotoxicity in Differentiated Y-79 Cells via Inhibition of ROS Generation and Ca(2+) Influx. *Int J Mol Sci*. 2016;17(7).
 15. Lv B, Huo F, Dang X, Xu Z, Chen T, Zhang T, et al. Puerarin Attenuates N-Methyl-D-aspartic Acid-induced Apoptosis and Retinal Ganglion Cell Damage Through the JNK/p38 MAPK Pathway. *J Glaucoma*. 2016;25(9):e792–801.

16. Wang C, Xie N, Zhang H, Li Y, Wang Y. Puerarin protects against beta-amyloid-induced microglia apoptosis via a PI3K-dependent signaling pathway. *Neurochem Res.* 2014;39(11):2189–96.
17. Zhu G, Wang X, Wu S, Li Q. Involvement of activation of PI3K/Akt pathway in the protective effects of puerarin against MPP⁺-induced human neuroblastoma SH-SY5Y cell death. *Neurochem Int.* 2012;60(4):400–8.
18. Tian F, Xu LH, Wang B, Tian LJ, Ji XL. The neuroprotective mechanism of puerarin in the treatment of acute spinal ischemia-reperfusion injury is linked to cyclin-dependent kinase 5. *Neurosci Lett.* 2015;584:50–5.
19. Zhang D, Ma G, Hou M, Zhang T, Chen L, Zhao C. The Neuroprotective Effect of Puerarin in Acute Spinal Cord Injury Rats. *Cell Physiol Biochem.* 2016;39(3):1152–64.
20. Wang N, Zhang Y, Wu L, Wang Y, Cao Y, He L, et al. Puerarin protected the brain from cerebral ischemia injury via astrocyte apoptosis inhibition. *Neuropharmacology.* 2014;79:282–9.
21. Zhou F, Wang L, Liu P, Hu W, Zhu X, Shen H, et al. Puerarin protects brain tissue against cerebral ischemia/reperfusion injury by inhibiting the inflammatory response. *Neural Regen Res.* 2014;9(23):2074–80.
22. Boulton M, Dayhaw-Barker P. The role of the retinal pigment epithelium: topographical variation and ageing changes. *Eye (Lond).* 2001;15(Pt 3):384–9.
23. Strauss O. The retinal pigment epithelium in visual function. *Physiol Rev.* 2005;85(3):845–81.
24. Yang P, Peairs JJ, Tano R, Jaffe GJ. Oxidant-mediated Akt activation in human RPE cells. *Invest Ophthalmol Vis Sci.* 2006;47(10):4598–606.
25. Yuan Y, Zong J, Zhou H, Bian ZY, Deng W, Dai J, et al. Puerarin attenuates pressure overload-induced cardiac hypertrophy. *J Cardiol.* 2014;63(1):73–81.
26. Li Z, Shangguan Z, Liu Y, Wang J, Li X, Yang S, et al. Puerarin protects pancreatic beta-cell survival via PI3K/Akt signaling pathway. *J Mol Endocrinol.* 2014;53(1):71–9.
27. Cantrell DA. Phosphoinositide 3-kinase signalling pathways. *J Cell Sci.* 2001;114(Pt 8):1439–45.
28. Liang D, Xiang L, Yang M, Zhang X, Guo B, Chen Y, et al. ZnT7 can protect MC3T3-E1 cells from oxidative stress-induced apoptosis via PI3K/Akt and MAPK/ERK signaling pathways. *Cell Signal.* 2013;25(5):1126–35.
29. Campochiaro PA. Retinal and choroidal neovascularization. *J Cell Physiol.* 2000;184(3):301–10.
30. Osborne NN, Casson RJ, Wood JP, Chidlow G, Graham M, Melena J. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. *Prog Retin Eye Res.* 2004;23(1):91–147.
31. Zhang J, Guo W, Tian B, Sun M, Li H, Zhou L, et al. Puerarin attenuates cognitive dysfunction and oxidative stress in vascular dementia rats induced by chronic ischemia. *Int J Clin Exp Pathol.* 2015;8(5):4695–704.
32. Tian F, Xu LH, Zhao W, Tian LJ, Ji XL. The neuroprotective mechanism of puerarin treatment of acute spinal cord injury in rats. *Neurosci Lett.* 2013;543:64–8.

33. Chipuk JE, Green DR. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol.* 2008;18(4):157–64.
34. Wang Y, Wang WL, Xie WL, Li LZ, Sun J, Sun WJ, et al. Puerarin stimulates proliferation and differentiation and protects against cell death in human osteoblastic MG-63 cells via ER-dependent MEK/ERK and PI3K/Akt activation. *Phytomedicine: international journal of phytotherapy phytopharmacology.* 2013;20(10):787–96.
35. Erickson KK, Sundstrom JM, Antonetti DA. Vascular permeability in ocular disease and the role of tight junctions. *Angiogenesis.* 2007;10(2):103–17.
36. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. *Progress in biophysics molecular biology.* 2003;81(1):1–44.
37. Garcia-Ramirez M, Villarroel M, Corraliza L, Hernandez C, Simo R. Measuring permeability in human retinal epithelial cells (ARPE-19): implications for the study of diabetic retinopathy. *Methods in molecular biology (Clifton NJ).* 2011;763:179–94.
38. Chen H, Wang H, An J, Shang Q, Ma J. Plumbagin induces RPE cell cycle arrest and apoptosis via p38 MARK and PI3K/AKT/mTOR signaling pathways in PVR. *BMC Complement Altern Med.* 2018;18(1):89.
39. Du L, Chen J, Xing YQ. Eupatilin prevents H₂O₂-induced oxidative stress and apoptosis in human retinal pigment epithelial cells. *Biomedicine pharmacotherapy = Biomedecine pharmacotherapie.* 2017;85:136–40.
40. Zha X, Wu G, Zhao X, Zhou L, Zhang H, Li J, et al. PRDX6 Protects ARPE-19 Cells from Oxidative Damage via PI3K/AKT Signaling. *Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology.* 2015;36(6):2217–28.
41. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell.* 1997;91(2):231–41.

Figures

(A)

(B)

Figure 1

Cytotoxicity effect of puerarin on normoxic ARPE-19 cells. (A) Safety dose of puerarin on ARPE-19 cells. ARPE-19 cells were treated with the indicated concentration of puerarin for 24 hours; cell viability was analyzed using WST-8 assays. The safety dosage of puerarin is $\leq 200 \mu\text{M}$ for 24 hours on normoxic ARPE-19 cells. (B) Biological effects of puerarin on normoxic ARPE-19 cells over time. ARPE-19 cells were treated with a noncytotoxic dose of puerarin for 1, 2, 5, 7 days. We assessed cell viabilities using WST-8 assays. Puerarin at doses $\leq 100 \mu\text{M}$ showed no cytotoxicity on normoxic ARPE-19 cells over time. The values shown represent the means $\pm$ SDs. The asterisk indicates $P < 0.05$ based on the Mann–Whitney U test.

Figure 2

Puerarin prevents hypoxia-induced apoptosis of ARPE-19 cells. (A) Time course of hypoxia-induced apoptosis and puerarin cyto-protection on hypoxic ARPE-19 cells according to WST-8 assay results. Most ARPE-19 cell deaths induced by hypoxia were observed after 8 hours of incubation in an anaerobic chamber. Addition of $50 \mu\text{M}$ and $100 \mu\text{M}$ of puerarin significantly protected ARPE-19 cells from hypoxia-induced cell death. (B) Representative images and quantitative results of DAPI, Annexin V-FITC, and PI triple fluorescence stainings under hypoxic conditions with puerarin treatment. DAPI (blue), Annexin V (green) and PI (red). Quantification of %Annexin V and % PI positivity in cells. Treatment with puerarin at $50 \mu\text{M}$ reduced the percentage of Annexin V-positive cells 3.78-fold ($P = 0.0004$) and the percentage of PI-positive cells 3.35-fold ($P = 0.0004$) compared with cells without treatment. Treatment with puerarin at

100 μM reduced the percentage of Annexin V positivity 4.31-fold ($P = 0.0003$) and that of PI positivity cells 3.46-fold ($P = 0.0002$) compared with the percentages in control cells in the hypoxia group. Scale bar = 20 μm . The values shown represent means $\pm$ SDs. The asterisk indicates $P < 0.05$ based on the Mann–Whitney U test.

Figure 3

Puerarin inhibits the hypoxia-induced disruption of barrier function on ARPE-19 cells. (A) Puerarin effects on the transepithelial electrical resistance (TER) of the ARPE-19 cell monolayer. At 24 hours after treatments, puerarin 50 μM and 100 μM showed protective effects on the hypoxia-induced TER reduction. (B) Puerarin effects on the tight junction protein expression of ZO-1 and Occludin in ARPE-19 cell monolayers. Western blot analysis of ARPE19 cells after treatment with puerarin and hypoxia exposure. Our quantitative results showed that 50 μM of puerarin significantly increased ZO-1 expression 1.99-fold ($p = 0.0148$) and Occludin protein levels 1.45-fold ($p = 0.0116$) in ARPE-19 cells exposed to hypoxia compared to the values in the hypoxia group. Puerarin at 100 μM also significantly increased ZO-1 expression 1.99-fold ($p = 0.018$) and Occludin protein levels 1.41-fold ($p = 0.02$) in ARPE-19 cells exposed to hypoxia compared to the levels in the hypoxia control cultures. (C) Immunocytochemistry of tight junction proteins (Zo-1, Occludin) on ARPE19 monolayers. Cells were treated with puerarin (50 μM , 100 μM) and exposed to hypoxia for 24 h, stained with DAPI (blue), ZO-1 (red), and Occludin (green) antibodies, and examined by fluorescence microscopy. Treatment with 50 and 100 μM puerarin prevented the disorganization of ZO-1 and Occludin induced by hypoxia. Scale bar = 20 μm . The values shown represent the mean $\pm$ SD. The asterisk indicates $P < 0.05$ using the Mann–Whitney U test. TER, transepithelial electrical resistance.

Figure 4

Puerarin mediated the Bcl2-regulated apoptotic pathway in ARPE-19 cells. (A) Levels of cleaved caspase 3, p-Bad, Bax, and Bcl-2 expression in ARPE19 cells evaluated by Western blot analysis. Treatment with puerarin (50 μ M, 100 μ M) significantly reduced hypoxia-induced p-Bad, Bax and cleaved caspase 3, and enhanced the antiapoptosis effects of Bcl-2 under hypoxic conditions. (B) Quantitative results of Western blot analyses expressed as a percentage of the control and represented as means $\pm$ SEMs. The values shown represent the means $\pm$ SDs. The asterisk indicates $P < 0.05$ based on Mann-Whitney U test results.

Figure 5

Puerarin activated the PI3K/Akt-dependent signaling pathway against hypoxia-induced cell apoptosis. (A) Western blot for p-Akt1 and p-ERK in a time-dependent manner under hypoxia in ARPE-19 cells. The expression of p-Akt decreased more than that of p-ERK after 24 hours of hypoxia. (B) Western blot analysis of p-PI3K, and p-Akt1. Puerarin treatment increased the levels of p-PI3K 1.66-fold ($P = .0015$), and that of p-Akt1 7.99-fold ($P < .0001$) under hypoxic conditions, compared with the control hypoxia group cells. Treatment with Akt inhibitor LY294002 significantly reduced the level of p-PI3K and pAkt1 compared to treatment with puerarin (50 μM). (C) Representative images and quantitative results of DAPI, Annexin V-FITC, and PI triple fluorescence stainings under hypoxia for 24 h (with puerarin and Akt inhibitor LY294002 treatments). Treatment with puerarin decreased Annexin-V and PI apoptosis signals; and, these effects were blocked by Akt inhibitor LY294002. DAPI (blue), Annexin V (green) and PI (red) stainings. Scale bar = 20 μm. The values shown represent means ± SDs. The asterisk indicates $P < 0.05$ based on Mann–Whitney U test results.