Abstract. Background: The role of epigenetic alterations in the pathogenesis of retinal degenerative diseases, such as macular degeneration is not well established. This study aimed to evaluate whether treatments with gamma-mangostin can rescue the hydrogen peroxide (H2O2)-induced cytotoxicity in human retinal pigment epithelial (ARPE-19) cells. Materials and Methods: ARPE-19 cells were treated with H2O2 alone or with gamma-mangostin plus H2O2 to investigate changes relating to cell viability, appearance of sub-G1 cells, antioxidant enzymes, and apoptotic-related proteins. Results: The data showed that under H2O2 treatment of 400 μM, there was a significant decrease in cell viability and enhanced apoptosis, together with an increased expression of Bax, Bad, cleaved-caspase-3, -8, and -9 at the protein level. On the contrary, the protein expression levels of Bcl2 and Bcl-xl were decreased. Gamma-mangostin pre-treatments (2-16 μM) could effectively prevent all alterations. Conclusion: Gamma-mangostin may conduct its eye-protective effects against H2O2-induced oxidative damage via anti-apoptotic and antioxidant mechanisms in ARPE-19 cells.

Across the retinal degenerative diseases, there are two typical pathological features, the chronic oxidative and inflammatory status. Among these retinal degenerative diseases, the age-related macular degeneration (AMD) is the most common cause of blindness among elderly citizens (1). Clinically, AMD will lead to progressive neurosensory macular destruction, which may cover the areas of retinal pigment epithelium (RPE), Bruch’s membrane, and choroid (2). AMD is cut into two stages: the early stage of AMD is characterized by the aberrant pigmentation of the RPE and the accumulation of extracellular deposits of lipid, cellular debris, and proteins (i.e., drusen), while the late (advanced) stage may manifest as non-exudative or exudative AMD (3, 4). In literature, AMD is one of the most investigated multifactorial eye diseases, and there have been many factors involved in the oxidative stress and inflammation of AMD (15).

To mimic the oxidative stress and damage, treatment with hydrogen peroxide (H2O2) is one of the most commonly used models both in vitro and in vivo (16, 17). However, the effect of H2O2 is very acute, occurs randomly, is dose-dependent and has multi-output effects, including cell proliferation, migration, survival, differentiation (18, 19) and...
incubator under a humidified 5% CO₂ supplement. These cells were previously described (27-29). First, to establish the H₂O₂-induced oxidative stress system, ARPE-19 cells (2×10⁵ cells/well) were treated with 0, 100, 200, 400 and 800 μM of H₂O₂ for 24 h, and then investigated with MTT assay. To examine the effects of Man against oxidative stress, ARPE-19 cells were pre-treated with indicated concentrations of Man for 24 h, followed by a 24-h exposure of H₂O₂. After any designed experiments, 10 μl of MTT solution (5 mg/ml) was added to each well and the cells were incubated at 37°C in the dark for another 4 h. The medium was then aspirated and 100 μl of DMSO was added and kept for exactly 10 min. Finally, the absorption was evaluated using a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland).

Flow cytometry analysis for cell apoptosis. Cell apoptosis was examined by Annexin V-FITC/PI methodology. ARPE-19 cells were grown in a six-well plate at a density of 2×10⁵ cells/well and treated with or without Man for 24 h, before treatment with H₂O₂ for 24 h. Then, the cells in all designed groups were washed twice with ice-cold PBS, resuspended in 300 μl of binding buffer, and stained with 10 μl of Annexin V-FITC plus 10 μl of PI in the dark for 20 min. After that, the stained cells were analyzed using the FACS Calibur instrument (BD Biosciences, San Jose, CA, USA). Morphological changes were photographed randomly as shown in Figure 1A-F.

Antioxidant enzyme measurements. Following treatment, the ARPE-19 cells were collected and three common antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-PX), were measured. Generally, SOD (Cat. 706002), CAT (Cat. 707002) and GSH-PX (Cat. 703002) activity were detected using commercially available assay kits (Cayman Chemical Company, Milpitas, CA, USA), following the manufacturers’ instructions.

Glutathione measurement. Following treatment, the ARPE-19 cells were harvested, and glutathione were measured using a commercially available assay kit from Sigma–Aldrich, Inc. All the procedures were carried out following the manufacturer’s instructions.

Materials and Methods

Reagents. Dulbecco’s Modified Eagle’s Medium (DMEM)/F12, penicillin/streptomycin, and certified fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich, Inc (St. Louis, MO, USA). Primary antibodies including anti-Bax (Cat. sc-7480), anti-Bad (Cat. sc-8044), anti-Bcl2 (Cat. sc-7382), anti-Bcl-xl (Cat. sc-8392), anti-uncleaved caspase-3 (Cat. sc-7272), anti-uncleaved caspase-8 (Cat. sc-56070), anti-uncleaved caspase-9 (Cat. sc-56076), anti-cleaved caspase-3 (Cat. sc-56052), anti-uncleaved caspase-8 (Cat. sc-81657), anti-uncleaved caspase-9 (Cat. sc-56073), anti-β-actin (Cat. sc-47778), and horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture conditions. Human retinal pigment epithelium (ARPE-19) cells were purchased from ATCC and routinely maintained in DMEM/F-12 medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin plus 100 μg/ml streptomycin in a steady 37°C incubator under a humidified 5% CO₂ supplement. These cells were passaged every 2 or 3 days and disposed after 15 passages.

MTT assay. Cell viability was evaluated using MTT assay as previously described (27-29). First, to establish the H₂O₂-induced oxidative stress system, ARPE-19 cells (2×10⁵ cells/well) were treated with 0, 100, 200, 400 and 800 μM of H₂O₂ for 24 h, and then investigated with MTT assay. To examine the influence of Man on the cell viability of ARPE-19 cells, cells was exposed to 0, 2, 4, 8 and 16 μM of for 24 h. To examine the effects of Man against H₂O₂-induced cytotoxicity, cultured ARPE-19 cells were pre-treated with indicated concentrations of Man for 24 h, followed by a 24-h exposure of H₂O₂. After any designed experiments, 10 μl of MTT solution (5 mg/ml) was added to each well and the cells were incubated at 37°C in the dark for another 4 h. The medium was then aspirated and 100 μl of DMSO was added and kept for exactly 10 min. Finally, the absorption was evaluated using a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland).

Results

Man protected ARPE-19 cells against H₂O₂-induced loss of cell viability. To establish an H₂O₂-induced cytotoxicity model, the cells were challenged with 0, 100, 200, 400 and 800 μM of H₂O₂ for 24 h. As shown in Figure 1H, 100, 200, 400 and 800 μM of H₂O₂ treatments caused 11.6%, 33%, 60.7% and 95.7% loss of cell viability, respectively (Figure 1H). At the same time, the treatments of 2, 4, 8 and 16 μM of Man did not cause a significant loss of cell viability.
Simultaneously, ARPE-19 cells treated with 100, 200, 400, 800 μM of H$_2$O$_2$, together with those treated with 16 μM of Man, were observed directly under the microscope. Consistently, H$_2$O$_2$ induced a loss of cell viability dose-dependently, while 16 μM of Man did not cause any obvious alteration in cell integrity or loss of viability (Figure 1A-F).

Interestingly, the pre-treatment of Man before H$_2$O$_2$ challenge can prevent the consequent damage and loss of cell viability in a dose-dependent manner (Figure 1I).

Man protected ARPE-19 cells against H$_2$O$_2$-induced apoptosis. To examine H$_2$O$_2$-induced ARPE-19 apoptosis, the cells were challenged with 0, 100, 200, 400 and 800 μM of H$_2$O$_2$ for 24 h. As shown in Figure 2A, 100, 200, 400 and 800 μM of H$_2$O$_2$ treatments induced 16.7%, 22.7%, 39.3, 75.7% of ARPE-19 cells to undergo apoptosis, respectively. The treatments of 2, 4, 8 and 16 μM of Man did not induce any significant apoptosis of ARPE-19 cells (data not shown). Meanwhile, 2, 4, 8 and 16 μM of Man pre-treatments could reduce the percentages of apoptotic cells induced by 400 μM of H$_2$O$_2$ dose-dependently (Figure 2B).

Man rescued H$_2$O$_2$-induced loss of antioxidant capacity in ARPE-19 cells. To explore the influence of H$_2$O$_2$ on antioxidant capacity in ARPE-19 cells, the cells were challenged with 400 μM of H$_2$O$_2$ for 24 h, and the status of
3 antioxidant enzymes, SOD, CAT and GSH-Px, together with GSH were measured. As shown in the figures, 400 μM of H2O2 treatment could decrease the activity of SOD, CAT and GSH-Px, in addition to the level of GSH (Figure 3A-D). At the same time, 16 μM of Man could enhance the activity of SOD and GSH-Px (Figure 3A and C), but not those of CAT or GSH (Figure 3B and D). Although to different degrees, 2, 4, 8 and 16 μM of Man were capable of rescuing the H2O2-induced loss of antioxidant status (Figure 3A-D).

Man reversed H2O2-induced alterations in apoptotic-related proteins of ARPE-19 cells. To confirm the influence of H2O2, and to check the reverse effects of Man on H2O2-induced alterations in ARPE-19 cells, the cells were pre-treated with various doses of Man, then challenged with 400 μM of H2O2 for 24 h, and the expression levels of apoptotic-related proteins were measured, including Bax, Bad, Bcl2, Bcl-xl, together with uncleaved and cleaved forms of caspase-3, -8, and -9. As shown in Figure 4, 400 μM of H2O2 treatment could enhance the levels of Bax, Bad (Figure 4A), cleaved caspase-3, -8 and -9 (Figure 4B). On the contrary, the levels of Bcl2 and Bcl-xl were decreased by the H2O2 challenge (Figure 4A), while the uncleaved form of caspase-3, -8 and -9 were unaltered. The pre-treatments of 4 and 16 μM Man could reverse the influences of H2O2 on Bax, Bad, Bcl2, Bcl-xl, cleaved caspase-3, -8, and -9 (Figure 4A and B).

Discussion

Although the precise pathogenesis of age-related macular degeneration (AMD) remains largely unclear, oxidative damage-induced loss of function in retinal pigment epithelium cells is thought to be the pathological cause in the initial stage of the AMD progress (30, 31). In addition, several lines of evidence have shown that reactive oxygen species-induced damage to retinal pigment epithelium cells are closely related to AMD (32, 33). Thus, in order to reveal the etiology of AMD and provide eye-protective strategy, the models of reactive oxygen species-induced damage to retinal pigment epithelium cells should be established for drug discovery and screening. To fulfill that, we have set up an H2O2-damage cell model in ARPE-19 cells.

Mangostins have been found to have antioxidant capacity (34, 35). In recent decades, alpha-mangostin has attracted most of the attention, which has been focused on its apoptosis-inducing capacity in colorectal cancer (36, 37). Following colorectal cell lines, a panel of cancer cell lines including breast and skin cancer cell lines, were also tested for validating its anti-cancer ability (38-42). In 2016, Fang and colleagues first found that in ARPE-19 cells, alpha-mangostin has protective effects on the cells in respect to oxidative-induced apoptosis (43). However, they did not investigate the effects of other mangostins (beta- and gamma-forms) and the effects of them on oxidative damage and apoptosis.

It is believed that reactive oxygen species-induced apoptotic cascades may play a critical role in AMD (44). From the same viewpoint, drugs which may improve the antioxidant capacity and do good to the mitochondrial integrity may have beneficial effects in fighting against AMD (45). Thus, we focused on the Bcl-2 family proteins which have anti-apoptotic (Bcl2 and Bcl-xl) and pro-apoptotic (Bax and Bad) properties, respectively (Figure 4A). In addition, the
The activation of caspase cascade is also critical; we have investigated the alterations about caspase-8, -9, and -3 (Figure 4B). Furthermore, we have checked the overall antioxidant status via measuring the alterations of SOD, CAT, GSH-Px, and GSH (Figure 3). We not only investigated the alterations after H$_2$O$_2$ treatment, but also the differences between with and without the pre-treatment of Man.

The highlights of the current study include that Man is effective in conducting its antioxidant capacity for the first time in ARPE-19 cells (Figure 1 and Figure 2). In addition, Man is capable of reversing H$_2$O$_2$-induced loss of antioxidant capacity of SOD, CAT, and GSH-Px (Figure 3). We not only investigated the alterations after H$_2$O$_2$ treatment, but also the differences between with and without the pre-treatment of Man.

The most important fact is that Man can be a strong antioxidant as alpha-mangostin was reported to protect ARPE-19 cells from oxidative stress (46). In our unpublished data, we have found that Man is more effective than alpha-mangostin in ARPE-19 cells (data not shown). Safety is another critical concern in clinical drugs. In the data of Chuang, 20 μM of alpha-mangostin caused a loss of cell viability by about 40% (46), while Man caused almost no loss of cell viability (Figure 1G) in the same ARPE-19 cell line. The oxidative stressor in our study is H$_2$O$_2$, while Chuang et al. used NaIO$_3$ (46).

In conclusion, the study has a solid and systematic set of results showing that Man effectively protected ARPE-19 cells against H$_2$O$_2$-induced stress via re-activating anti-apoptotic and antioxidant mechanisms. These findings polish the potential of Man as a novel drug for AMD and other diseases. Further investigations, such as those in mice or rat models, are needed for a better understanding of the underlying mechanisms and possible clinical practice.

Figure 3. The effects of H$_2$O$_2$ and Man on antioxidant status of ARPE-19 cells. Pre-treatments of 0~16 μM Man for 24 h to ARPE-19 cells before challenged by 400 μM H$_2$O$_2$ for 24 h. Then the activity of SOD (A), CAT (B), GSH-Px (C), and the relative amounts of GSH (D) were measured. Data are presented as mean±SD of at least three experiments. *Statistically significant (p<0.05) compared with the untreated group. †Statistically significant (p<0.05) compared with the 400 μM H$_2$O$_2$ alone group.
Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

Authors’ Contributions

Hu PS, Hsia NY and Chien WC conceived and designed the experiments. Mong MC, Wang YC and Chang WS performed the experiments. Hsia TC Chang HM and Tsai CW analyzed the data. Hu PS and Hsia NY contributed with reagents, materials and analysis tools. Tsai CW and Bau DT wrote and revised the article.

Acknowledgements

The study has been supported by Chang Bing Show Chwan Memorial Hospital (BRD-109011), China Medical University Hospital (DMR-110-117), Asia University plus China Medical University (CMU110-ASIA-05) and Changhua Christian Hospital (109-CCH-IRP-113).

References


12 Hu et al: Gamma-mangostin Against H2O2-induced Oxidative Damage in ARPE-19 Cells


Received April 2, 2022
Revised April 26, 2022
Accepted April 27, 2022