ISSN : 0974 - 7435 Volume 10 Issue 18

2014

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 10(18), 2014 [10191-10196]

Protective effects of polygonum multiflorum polysaccharide against H_2O_2-induced apoptosis in PC12 cells

Yatao Xu, Xiuhong Jiang, Shumei Wang, Shengwang Liang*
Department of Chemistry and Analysis of Traditional Chinese Medicine, Traditional Chinese Medicine College, Guangdong Pharmaceutical University, Guangzhou 510006, P.R. (CHINA)
Phone : 020-39352172
E-mail : swliang371@163.com

ABSTRACT

Objective To investigate the protective effects of Polygonum Multiflorum polysaccharide (PMP) on H_2O_2-induced apoptosis in PC12 cells. Methods MTT assay was used to assess the viability of PC12 cells which exposed to PMP with gradient concentrations; Flow cytometry(FCM) assay was used to analyze the apoptosis ratio of PC12 cells; Hoechst 33258 stain assay was used to observe the changes in PC12 cells nuclear morphology; Results PMP could protect the PC12 cells morphology, reduce the apoptosis rate and rescue H_2O_2-induced changes in nuclear morphology. Conclusion These results indicated that PMP could protect PC12 cells from H_2O_2-induced damage.

KEYWORDS

H_2O_2; PC12 cells; Polygonum multiflorum polysaccharide (PMP); Apoptosis.

© Trade Science Inc.

INTRODUCTION

The *Polygonum Multiflorum* Thunb. is a traditional Chinese medicine, which has the function of tonifying liver and kiney, strengthening bones and muscles, nourishing blood and blackening hair. Meanwhile, it also can ease the aging and fall hematic fat[1-3]. Aging is one that multiple organ progressive failure with age, and its performance in cell aging and decline in physiological function. However, the aging mechanism is not clear, there are many theories, such as free radical mechanism, telomere mechanism, apoptosis mechanism and so on. Apoptosis is involved in many pathological processes associated with aging, such as osteoporosis, Alzheimer's disease. At present, the effect of the apoptosis has become a hotspot in research at home and abroad[4,5].

The research established PC12 cells damage model by H_2O_2, the MTT, FCM and Hoechst33258 fluorescence assay were used to explore the apoptosis rate and evaluate the protective effects of the PMP in cells injured. Thereby providing meaningful pharmacology basis for the thorough study of anti-aging effect of Polygonum multiflorum.

MATERIALS AND METHODOLOGY

Materials

Polygonum Multiflorum polysaccharide was extracted and separated by our laboratory. PC12 Cells were gifted by New drug screening and evaluation center of Guangdong Pharmaceutical University (Guangzhou,China). Annexin V-fluorescein isothiocyanate(FITC) apoptosis detection kit, PI(propidium iodide), Hoechst33258 were purchased from Kaiji Institute of Biological Engineering (Nanjing,China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), Trypsin were obtained from Gibco.

Methodology

PC12 cells culture and treatment[6-8]

On the 37℃, 5%CO_2 saturated humidity condition that PC12 cell were treated in the 10% fetal bovine serum of DMEM in 2 to 3 days, and replaced the culture media in order to maintain the good growth state. The cells' were digested by 0.25% trypsin and subcultured when it attachment rate was about 80%. At the logarithmic phase, the well-grown PC12 cells were plated on 96-well plates at a density of 1×10^5 cell/mL. After cultured with the serum-deprived medium for 24 h, PC12 cells were pretreated with various concentrations (0.625, 1.25, 2.5 and 5 mg·mL^{-1}) of PMP for 24 h,and then exposed to H_2O_2 (1.25 mM) for 6 h[9]. Control cells were grown in DMEM without PMP and H_2O_2. H_2O_2 injury cells were exposed to H_2O_2 (1.25 mM) alone. The measurements were performed after cells were damaged by H_2O_2 for 6 h.

Cell viability assay[10-12]

The well-grown PC12 cells in 96 well plates were treated with various concentrations (0.625, 1.25, 2.5 and 5 mg·mL^{-1}) of PMP for 24 h, then exposed to H_2O_2 (1.25 mM) for 6 h at 37℃, 6 parallel holes were set in every group. MTT solution was added into each well to make final concentration of 0.5 mg·mL^{-1}, and the reaction mixture was allowed to incubate at 37℃ for 4 h followed by the addition of 150 μL DMSO to dissolve the formazan product. After stirring for 10 min on a microtiter plate shaker, the absorbance was measured at 490 nm using a microplate reader. Cell viability was expressed as the percentage of the value in control cultures.

Flow cytometry (FCM)[13-15]

At the end of treatment, all cells were digested and collected. The concentration was adjusted to 1 x 10^6 cells/mL. Then 100 μL cell suspension, 5 μL Annexin V-FITC and 10 μL PI were added in 5 mL flow cytometry tube, after blended, the cells were stained for 15 min at room temperature away from light, then 400 μL PBS were added and the cells were analyzed with flow cytometry immediately. The excitation wavelength was 488 nm, 1 x 10^6 cells were counted. All data were processed by Cell Quest software.

Hoechst 33258 staining[14-16]

After treatment with PMP and/or H_2O_2, PC12 cells were examined by Hoechst 33258 staining. the culture medium was removed and the PC12 cells were fixed with 4% paraformaldehyde for 20 min. After rinsing twice with PBS, the nuclear were stained with 1 mg·mL^{-1} Hoechst 33258 for 30 min at room temperature in the dark, and then examined by fluorescence microscopy.

Statistical analyses

Data were presented as mean±SD. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test. $p<0.05$ was considered to indicate statistical significance.

RESULTS

MTT assay analysis for H_2O_2-induced PC12 cell viability

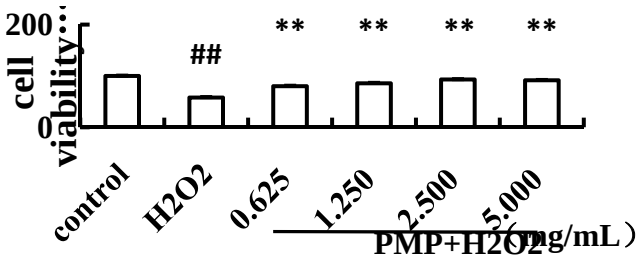
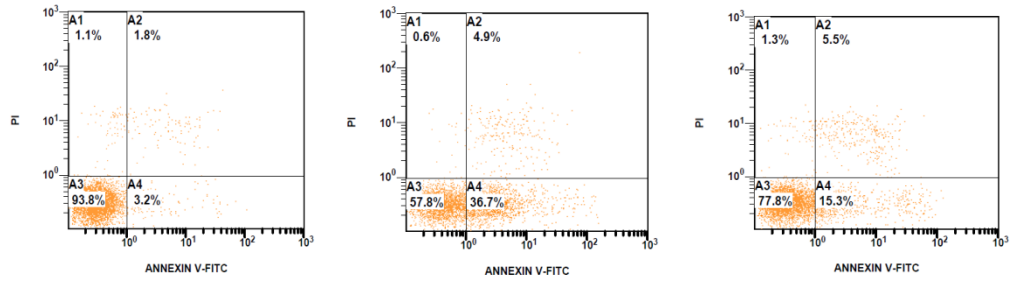


Figure 1 : Effects of PMP on H_2O_2-induced PC12 cell viability by MTT assay. After the treatment of cells with different concentrations of PMP (0.625, 1.25, 2.5 and 5 mg·mL^{-1}) for 24 h and/or H_2O_2 (1.25mM) for 6 h, cell viability was assessed by MTT assay. $^{\#\#}P$ 〈0.05 *vs* control group, $^{**}P$ 〈0.05 *vs* model group.

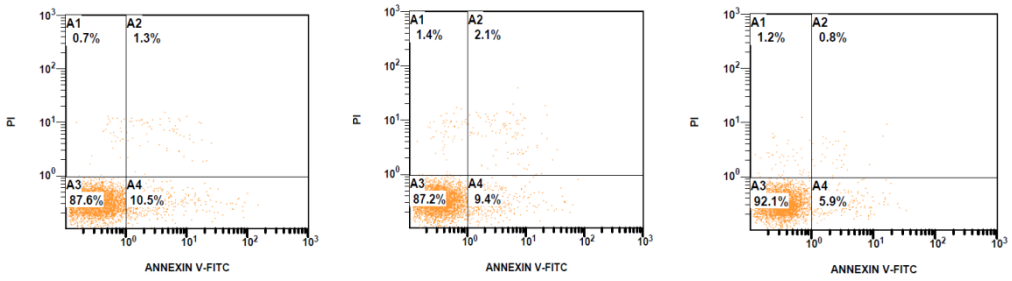
As shown in Figure 1, PC12 cells activity was significantly decreased to 58±4% after injured by H_2O_2(1.25 mM) alone for 6 h. Compared with control cells, Pretreatment with different concentration of PMP (0.625, 1.25, 2.5 and 5 mg·mL^{-1}) improved the cells viability. The result showed PMP has a protective effect on PC12 cells, and with a maximum viability at 2.5 mg·mL^{-1}.

Flow cytometry (FCM) analysis for H_2O_2-induced apoptosis in PC12 cells

Flow cytometry instrument analysis results show that the apoptosis rate of control cells were 3.2%; On the contrary, treatment of cells with H_2O_2(1.25 mM) alone significantly increased apoptotic rate to 36.7%. Pre-treatment with PMP, the percentage of apoptosis cells was significantly decreased, and with the increase of polysaccharide concentrations, the apoptosis rate fell from 15.3% to 5.9%, and significantly lower than the model group.



Control H_2O_2 H_2O_2+PMP(0.625 mg·mL^{-1})



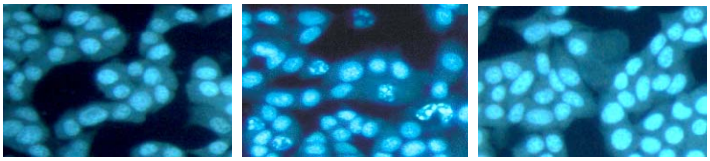
H_2O_2+PMP(1.25 mg·mL^{-1}) H_2O_2+PMP(2.5 mg·mL^{-1}) H_2O_2+PMP(5 mg·mL^{-1})

Figure 2 : Effects of PMP on H_2O_2-induced apoptosis PC12 cells by FCM. cells treated with PMP(0.625, 1.25, 2.5 and 5 mg·mL^{-1}) for 24 h, then exposed to H_2O_2 (1.25 mM) for 6 h, Cell apoptosis was tested by flow cytometry.

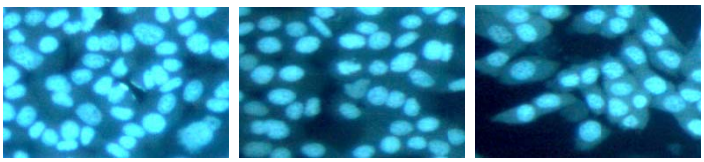
Effects of PMP on fluorescent staining in H_2O_2-induced PC12 cells

The control PC12 cells had oval or round-shaped nuclei with homogeneous shallow blue fluorescence, and the chromatin distribution uniformity. In contrast, the nuclei of H_2O_2-induced PC12 cells became shriveled or the fluorescence

intensity increased. the cells appeared typical apoptotic morphological changes that nuclei cracked into pieces and formed apoptotic bodies, ect. These changes were prevented significantly by pretreatment of PC12 cells with PMP (0.625, 1.25, 2.5 and 5 mg·mL^{-1}), with increasing concentration of polysaccharides, the apoptosis rate of cells decreased, especially at concentrations of 2.5 mg·mL^{-1} (Figure 3E) and 5 mg·mL^{-1} (Figure 3F).



A B C



D E F

Figure 3 : Effects of PMP on H_2O_2-induced fluorescent staining with Hoechst 33258 in PC12 cells (×200). A:control cells; B:cells treated H_2O_2(1.25 mM) alone for 6 h; C-F:cells pre-incubated with different concentration of PMP(0.625, 1.25, 2.5 and 5 mg·mL^{-1}) for 24 h and exposed to H_2O_2(1.25 mM) for 6 h.

CONCLUSIONS

In recent years, the generation of reactive oxygen species and the study to damage mechanism of biological molecules were widely concerned in various disciplines. The occurrence of many diseases are closely related with the involvement of reactive oxygen species. H_2O_2 is an important active oxygen, and exogenous H_2O_2 can easily enter the cell through the cell membrane. The presence of transition metals in cells was carried out by Fenton reaction. Then forming highly reactive free radicals, such as a single peptide oxygen, hydroxyl radicals, causing further damage to the cells. Due to the operation of H_2O_2 injury model is very simple and easy to control, so it was commonly used in vitro cell simulation of oxidative damage[17].

PC12 cells, a cell line cloned from rat pheochromocytoma, on the biological characteristics of the cells and the occurrence of certain neurons are derived from neural crest cells. Differentiated PC12 cells have certain properties of neurosecretory cells and neuronal cells. Enzymes, membrane receptors and neurotransmitter synthesis in differentiation of PC12 cells are similar with midbrain dopamine neurons. So PC12 cells can simulate the normal function of the neurons[18,19]. According to the experimental needs to control cell growth conditions and the environment, we can observe the cellular morphological and biochemical changes. Furthermore, relative to the generation of nerve cells in primary culture, PC12 cells, although not as neurons that directly reflect the pathological and physiological conditions of the central nervous system, but the PC12 cells are passaged cells, with a very high stability and homogeneity, with infinite passage and other advantages. In the study of drug mechanism, application of PC12 cells can eliminate the effect of hybrid culture of other cells, and the experimental conditions easy to control. So PC12 cells are widely applied to the study of nervous system diseases.[20,21]

MTT is a common monitoring indicators which is usually used to evaluate cell's survival, growth status and degree of injury. The amount of MTT formazari is proportional to the activity of succinate dehydrogenase that exists in living cells[22]. That is to say, the bigger the OD value, the smaller the cell damage. In this study, the anti-apoptosis roles of PMP were investigated by MTT assay, According to results, PMP could be against the PC12 cells morphological changes in a certain extent which induced by H_2O_2, PMP also can alleviate the damage of cell membrane, enhance the vitality of cells, increase the cell survival rate and reduce the cell death rate induced by oxidative damage. However, The effective dose of PMP and the mechanism of action in vivo need pending further study.

Hoechst33258 is a specific kind of DNA dye, which can through the cell membrane and combine with DNA A2T key, It will emit blue fluorescence under UV excitation. According the nuclear size, shape, chromatin states and fluorescence intensity to determine whether a cell apoptosis[23], Hochest33258 staining results showed that PMP had protective effect on PC12 cell injury induced by hydrogen peroxide. When compared with the H_2O_2-induced PC12 cells, polysaccharide group cells had less shrunken nuclei, more shallow blue fluorescence, and the apoptosis was ameliorated.

AnnexinV/PI method can not only detect early apoptosis, but also distinguish the cell apoptosis and death. It saves time and effort, because this method does not require fixed. Therefore, AnnexinV/PI method is the preferred method of quantitative detection of apoptosis by flow cytometry. Flow cytometry by AnnexinV / PI double staining was measured

apoptosis rate. Compared H_2O_2-induced PC12 cells, the apoptosis rate of polysaccharide protection group cells was obviously droped, showing the antiapoptotic effect in vitro of PMP.

In summary, the polysaccharide from *Polygonum multiflorum* Thunb on H_2O_2-induced nerve damage in PC12 cells have a protective effect. However, the mechanisms that underline the protective effect have not been fully understood, it remains to be further studied.

CONFLICT OF INTEREST

None of the authors have any potential conflicts of interest.

ACKNOWLEDGMENTS

Yatao Xu and Xiuhong Jiang made substantial contributions to this experimental. Xiuhong Jiang made contributions to experimental scheme and data acquisition, Yatao Xu was mainly responsible for the analysis of data and writing the manuscript, Shumei Wang mainly for the direction and supervision of the entire experiment. Shengwang Liang participated in the supervision of the research group, acquisition of funding, and the guidance of data analysis and important content of the audit revision. All authors were reviewed and approved the final manuscript.

Furthermore, We are grateful to Professor Linquan Zang from the Guangdong Pharmaceutical University, China for help with experimental guidance, and thank Professor Yisheng Zhou and Associate Professor Feng Wang for their helpful suggestions in preparing this manuscript.

REFERENCES

[1] X.Yang; Effect of ethanolic extract from root tuber of *polygonummultif lorum* Thunb on liver and brain monoamine oxidase in senescenceaccelerated mice in vivo. China Journal of Chinese Materia Medica, **21**, 48–49 **(1996)**.

[2] Y.C.Chan, M.F.Wang, H.C.Chang; Polygonum multiflorum extracts improve cognitive performance in senescence accelerated mice. Am J Chin Med, **31(2)**, 171–179 **(2003)**.

[3] X.Li, K.Matsumoto, Y.Murakami; Neuroprotective effects of Polygonum multiflorum on nigrostriatal dopaminergic degeneration induced by paraquat and maneb in mice. Pharmacol Biochem Behav, **82**, 345–352 **(2005)**.

[4] E.Dale Bredesen, V.Rammohan Rao, Patrick Mehlen; Cell death in the nervous system. Nature, **443(7113)**, 796–802 **(2006)**.

[5] N.Inna Lavrik, Roland Eils, Nicolai Fricker; Understanding apoptosis by systems biology approaches. Mol Biosyst, **5**, 1105–1111 **(2009)**.

[6] Meng Jin-Lan, Mei Wei-Yi, Dong Yan-Fen; Heat shock protein 90 mediates cytoprotection by H2S against chemical hypoxia-induced injury in PC12 cells. Clin Exp Pharmacol Physiol, **38(1)**, 42–49 **(2011)**.

[7] Yu Shu, Liu Mei, Gu Xiao-Song; Neuroprotective Effects of Salidroside in the PC12 Cell Model Exposed to Hypoglycemia and Serum Limitation. Cell Mol Neurobiol, **28(8)**, 1067–1078 **(2008)**.

[8] Chen Xiao-Hong, Chen Shao-Qiong, Jiang Ying; Rongbiao Pi. Minocycline reduces oxygen–glucose deprivation-induced PC12 cell cytotoxicity via matrix metalloproteinase-9,integrin b1 and phosphorylated Akt modulation. Neurol Sci, **34(8)**, 1391–1396 **(2013)**.

[9] O.Irina Zakharova, V.Tatyana Sokolova; α-Tocopherol at Nanomolar Concentration Protects PC12 Cells from Hydrogen Peroxide-Induced Death and Modulates Protein Kinase Activities. Int. J. Mol. Sci, **13(9)**, 11543–11568 **(2012)**.

[10] T.Mosmann; Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, **65**, 55–63 **(1983)**.

[11] He Jin-Ting, Mang Jing, Mei Chun-Li; Neuroprotective Effects of Exogenous Activin A on Oxygen-Glucose Deprivation in PC12 Cells. Molecules, **17**, 315–327 **(2012)**.

[12] Marta Pera, Pelayo Camps, Diego Mun˜oz-Torrero; Undifferentiated and Differentiated PC12 Cells Protected by Huprines Against Injury Induced by Hydrogen Peroxide. PLoS ONE, **8(9)**, e74344 **(2013)**.

[13] M.Ogawa, T.Yamaguchi, A.Setiyono; Some characteristics of a cellular receptor for virulent infectious bursal disease virus by using flow cytometry. Arch Virol, **143**, 2327–2341, **(1998)**.

[14] Lan Ai-Ping, Liao Xin-Xue, Mo Li-Qiu; Hydrogen Sulfide Protects against Chemical Hypoxia-Induced Injury by Inhibiting ROS-Activated ERK1/2 and p38MAPK Signaling Pathways in PC12 Cells. PLoS ONE, **6(10)**, e25921 **(2011)**.

[15] Tao Li-Zhen, Li Xiao-Feng, Zhang Ling-Ling; Protective effect of tetrahydroxystilbene glucoside on 6-OHDA-induced apoptosis in PC12 cells through the ROS-NO pathway. PLoS ONE, **6(10)**, e26055 **(2011)**.

[16] Jiang Hong, Lv Peng-Fei, Li Ji-Chang; Baicalin inhibits colistin sulfate-induced apoptosis of PC12 cells. Neural Regen Res, **8(28)**, 2597–2604 **(2013)**.

[17] W.G.Li, J.Francis, V.A.Morais; H_2O_2-induced superoxide anon production by a non-phagocytic NAD(P) H oxidase cause oxidant injury. J Biolehem, **276(31)**, 29251–29256 **(2001)**.

[18] A.P.Betrosian, F.Frantzeskaki, A.Xanthaki; Efficacy and safety of high-dose ampicillin/sulbactam vs. colistin as monotherapy for the treatment of multidrug resistant Acinetobacter baumannii ventilator-associated pneumonia. J Infect, **56(6)**, 432–436 **(2008)**.

[19] Qu Li-Na, Chen Hai-Long, Liu Xin-Min; Protective effects of flavonoids against oxidative stress induced by simulated microgravity in SH-SY5Y cells. Neurochem Res, **35(9)**, 1445–1454 **(2010)**.

[20] H.Meng, C.LiC, L.Feng; Effects of Ginkgolide B on 6-OHDA-induced apoptosis and calcium over load in cultured PC12. Int J Dev Neurosci. **25(8)**, 509–514 **(2007)**.

[21] Naoko Sasaki, Takeshi Toda, Takao Kaneko; Flavonoids suppress the cytotoxicity of linoleic acid hydroperoxide toward PC12 cells. Biol. Pharm. Bull. **25(8)**, 1093–1096 **(2002)**.

[22] M.V.Berridge, A.S.Tan; Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Archives Biochem Biophys, **303(2)**, 474–482 **(1993)**.

[23] K.Ding, J.N.Fang, T.X.Dong; Characterization of a Rhamnogalacturonan and Xylogluean from Neriumindicum and Their Activities on PC12 Pheochr-omocyton1a Cells. Natural Products, **66(1)**, 7-10 **(2003)**.