International Journal of Clinical Medicine Research
2018; 5(3): 55-60
http://www.aascit.org/journal/ijcmr
ISSN: 2375-3838

AASCIT American Association for Science and Technology

# Snail Extracts Protect Neuroblasts SH-SY5Y Cells from Damage Induced by Hydrogen Peroxide: A Pilot Study

**Zhao-hui Zhang[1], Ping Wang[2], Qiao-hong Ji[1], Ying-li Duan[2], Shou-min Xi[2, *]**

[1]Department of Pathology and Pathophysiology, Henan University of Science and Technology, Luoyang, PR China
[2]The Key Laboratory of Pharmacology and Medical Molecular Biology, Henan University of Science and Technology, Luoyang, PR China

## Email address

xishoumin1968@163.com (Shou-min Xi)
[*]Corresponding author

## Citation

Zhao-hui Zhang, Ping Wang, Qiao-hong Ji, Ying-li Duan, Shou-min Xi. Snail Extracts Protect Neuroblasts SH-SY5Y Cells from Damage Induced by Hydrogen Peroxide: A Pilot Study. *International Journal of Clinical Medicine Research.* Vol. 5, No. 3, 2018, pp. 55-60.

**Received**: March 18, 2018; **Accepted**: March 30, 2018; **Published**: May 16, 2018

**Abstract:** The snail serves as a well-documented traditional Chinese medicine for many diseases. However, there has been no report for the potential benefit, especially the neuroprotective effect of the snail polypeptide mixtures (SPM). We made preliminary attempt to explore the protective effect of SPM against cell damage induced by $H_2O_2$ in neuroblast-drived cell line SH-SY5Y. In the present study, $H_2O_2$ at the concentration of 1.54 mmol/L significantly induced nuclear condensation, MMP dissipation and decreased expression of PCNA and BDNF. SPM at the concentartion of 39 mg/L and 156 mg/L improved morphological changes of nucleus and MMP dissipation induced by $H_2O_2$, which was associated with significantly increased expression of PCNA and BDNF. SPM could protect SH-SY5Y cells from apoptosis induced by $H_2O_2$, which holds promise as a novel drug for the treatment of neurodegenerative diseases.

**Keywords:** Snail Polypeptide, Hydrogen Peroxide, SH-SY5Y Cell, Apoptosis

## 1. Introduction

It has been widely demonstrated that oxidative stress plays an important role in many neurodegenerative diseases [1, 2]. Oxidative injury is induced by reactive oxidative species (ROS) which attack bio-macromolecules, such as proteins, DNA and lipids. One of the most characterized ROS, hydrogen peroxide ($H_2O_2$) is generated in redox processes, and a major mediator of oxidative stress, which could lead to apoptosis in a variety of cell-types by lipid peroxidation as well as protein and DNA oxidation [3]. Moreover, $H_2O_2$ has also been suggested to be a messenger in intracellular signaling cascades [4].

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophic family and probably the most abundant neurotrophic factor in the central nervous system, and contributes to neuronal survival, growth and differentiation [5]. BDNF plays an important role in long-term potentiation and memory formation [6]. The deficiency in the regulation of BDNF are involved in a number of neurodegenerative and neuropsychiatric disorders [7].

Proliferating cell nuclear antigen (PCNA) plays an important role in DNA synthesis, especially in triggering cell proliferation [8]. It has been shown that PCNA is a processivity factor for DNA polymerases delta and epsilon, and is essential for both DNA replication and repair [9]. PCNA is required in the resynthesis step of nucleotide excision repair. In quiescence fibroblasts, the amount of nuclear-binding PCNA increases in a dose dependentmanner when treated with methyl methane sulfonate and $H_2O_2$, suggesting that PCNA might be involved in post-damage DNA repair [10]. An *in vivo* study by Okada *et al* showed that the exposure of old mice to UV light for one week result in the increase of PCNA in pyramidal cells, suggesting that the expression of PCNA might also be involved in DNA damage and repair in neurological organs [11].

*Achatina fulica* is a special of large land snail with soft body which is rich in proteins such as snail polypeptides (SP), vitamins and polysaccharides. The species serves as a popular food worldwide, and also a well-documented traditional Chinese

medicine for many diseases such as diabetes and hypertension. However, there has been no report for the potential benefit, especially the neuroprotective effect of the snail polypeptide mixtures in experimental setting. In the present study, we made preliminary attempt to explore the protective effect of SPM extracted from *Achatina fulica* against cell damage induced by $H_2O_2$ in neuroblast-drived cell line SH-SY5Y.

## 2. Materials and Methods

### 2.1. Reagents

*Achatina fulica* was purchased from Luoyang Lver Agricultural Science and Technology Co. Ltd. (Henan, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and Rhodamine123 were purchased from Sigma (St. Louis, MO, USA). DMEM medium and fetal calf serum were purchased from GIBCO Chemical Co (Grand Island, NY. USA). Mouse monoclonal antibody against human PCNA (BM0104) and rabbit polyclonal antibodies against BDNF (BA05651 and BA0665-2, for IH and WB, respectively) were purchased from Wuhan Boster Bio-engineering Limited Company (Hubei, China). Streptavidin-Peroxidase Immunohistochemistry kit (ZYMED, USA) was purchased from Bioss (Beijing, China). DAB Horseradish Peroxidase Color Development Kit was purchased from Dingguo Changsheng Biotechnology Co. Ltd. (Beijing, China). All other chemicals were of analytical grade and commercially available.

### 2.2. Purification of Snail Polypeptide

Fresh raw tissues of *Achatina fulica* (200g) were cut into small pieces, and then collected into tubes with 500 mL of precooled acetic acid solution (pH 3.5). After homogenization with a colloidal mill, the supernatant was obtained by centrifugation at 5500 rpm for 10 min at 4°C. The purification of snail polypeptide was performed as described previously [12].

### 2.3. Cell Culture

SH-SY5Y cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated newborn calf serum and 2 mM glutamine in a humidified atmosphere with 5% $CO_2$ at 37°C. The cells were sub-cultured every 2 days.

### 2.4. Determination of Cell Viability by MTT Assay.

Cell viability was determined by measuring the dehydrogenase activity retained in the cultured cells through the MTT assay. The cells were plated at a density of $4\times10^4$ cells/mL in 96-well plates for 8 replicates and incubated for 1 day. The cells were then incubated for 24 h in fresh medium without newborn calf serum in the presence of SPM for 24 h before the treatment with $H_2O_2$ for another 24 h. The cells were then incubated with 5 mg/mL MTT (25 μL/well) in 10% FBS DMEM medium for 4 h at 37°C. The MTT-containing medium was removed and the intracellular purple was dissolved in DMSO for quantification at 490 nm with a microplate reader.

### 2.5. Assessment of Apoptosis by Hoechst 33342 Staining

For detection of apoptosis, Hoechst 33342 staining was employed to visualize the apoptotic nucleus. After treatment, cells were washed three times with phosphate-buffered saline (PBS), and stained with 5 μg/mL Hoechst 33342 in PBS at 37°C for 30 min. Apoptotic cells were characterized by the condensed or fragmented nuclei, as visualized using a fluorescence microscope (Olympus, Melville, NY, USA).

### 2.6. Mitochondrial Membrane Potential Change

To determine mitochondrial membrane potential change, the experimental SH-SY5Y cells were stained with Rh-123, a cationic fluorescent dye. Briefly, SH-SY5Y cells were cultured in a Petri dish, after each treatment cells were stained with Rh-123 (10 M) in PBS for 30 min at room temperature then washed three times with PBS, the cells were observed with a fluorescence microscope, and the fluorescence intensity of at least eight fields per dish was analyzed with an Alpha Ease FC imaging system (Alpha Imager 2200, Alpha Innotech).

### 2.7. Morphological Analysis and Fluorescence Immunohistochemistry Staining

Glass cover slips were placed in 6-well plates (Greiner Bio-One, Germany), and cells were seeded onto the cover slips. After treatment, the cover slips were fixed with 4% paraformaldehyde and then washed 3 times with 0.01 M PBS. After that, the cover slips were incubated with 0.5% Triton X-100 in PBS for 15 min at 22°C and washed with PBS. After incubation with primary antibodies overnight at 4°C, the cover slips were incubated with secondary antibodies. Stained cells were mounted and imaged by using a Nikon inverted microscope. Densitometry analysis was performed with Image pro-plus 6.0 software.

### 2.8. Western Blot Analysis

Whole cell extracts were prepared in lysis buffer (50 mM HEPES, pH 7.4, containing 150 mM NaCl, 1% TritonX-100, 0.4% SDS, 5 mg/ml aprotinin, 5 mg/ml leupeptin, 1mM PMSF, 1 mM DTT, 1 mM $Na_3VO_4$, and 1 mM NaF). After centrifugation at 3,000 g for 20 min, the supernatants were collected for western blot analysis. The total protein concentration of the supernatants was determined with Lowry method. Equal amounts of protein were electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose membranes. Non-specific binding was blocked with 5% BSA in TBST (20 mM Tris-HCl, pH 7.5, containing 137 mM NaCl, and 0.1% Tween 20). The membranes were then incubated with antibody for PCNA or BDNF at 4°C overnight. followed by incubated with anti-mouse IgG conjugated to horseradish peroxidase for 1.5 h at room temperature. The blots were developed using the

ECL detection system with β-actin as the internal standard.

### 2.9. Statistical Analysis

All of the data are expressed as the mean ± standard deviation (SD). The values obtained before and after treatment within each group were analyzed using paired Student's t test. All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All *P* values were 2 sided, and $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. The effect of $H_2O_2$ on the Cell Viability of SH-SY5Y Cells

As can be seen from Figure 1, the viability of SH-SY5Y cells decreased in a dose-dependent manner when treated with 0.25-6.0 mM $H_2O_2$ for 24 h- The $IC_{50}$ of $H_2O_2$ in SH-SY5Y cells was 1.54 mM as calculated from the dose-response curve. Based on the results of MTT assay, the cells were further treated with 1.54 mM $H_2O_2$ for 24 h and the results showed a 47.65 ± 3.52% loss of cell viability (Figure 1). Pretreatment with SPM for 24 h at the concentration ranging from 39 $mg \cdot L^{-1}$ to 1250 $mg \cdot L^{-1}$ significantly reduced the cell death ($P < 0.05$) induced by $H_2O_2$, and the effect of SPM was in a dose-dependent manner (Figure 2).

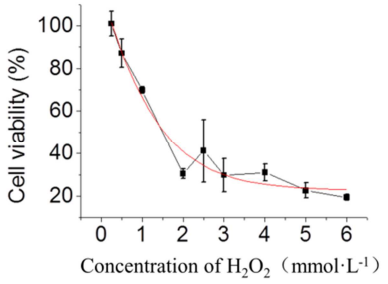


***Figure 1.*** *The effect of $H_2O_2$ on the viability of SH-SY5Y cells.*

SH-SY5Y cells were treated with $H_2O_2$ at indicated concentration for 24 h, and cell viability was measured by MTT assay.

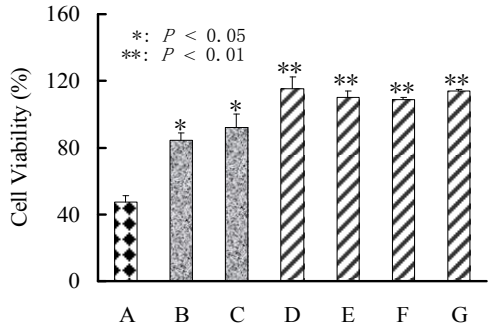


***Figure 2.*** *Pretreatment with SPM protects SH-SY5Y cell from death induced by $H_2O_2$. Cell were pretreated with SPM at indicated concentration for 24h, followed by 1.54 mM $H_2O_2$ treatment for 24h. Cell proliferation was measured by MTT assay. A: $H_2O_2$; B:39mg·L-1 SPM followed by $H_2O_2$; C:78 mg·L-1 SPM followed $H_2O_2$; D:156 mg·L-1 SPM followed $H_2O_2$. E:312.5 mg·L-1 SPM followed $H_2O_2$. F:625 mg·L-1 SPM followed $H_2O_2$. G:1250 mg·L-1 SPM followed $H_2O_2$, *P<0.05, **P<0.01 compared with H2O2 group.*

### 3.2. The effect of SPM on the apoptosis of SH-SY5Y cells induced by $H_2O_2$

We chose 39 mg·L-1 as a low dose SPM (SPM-L) and 156 mg·L-1 as a high dose SPM (SPM-H) to evaluate the effect on viability of SH-SY5Y cells visualized with Hoechst 33342 staining. As shown in Figure 3, the control cells showed intact nuclei. Incubation of SH-SY5Y cells with 1.54 mM $H_2O_2$ for 24 h resulted in perinuclear chromatin condensation and especially nuclear fragmentation, which are characteristics of apoptotic nuclei. These apoptotic changes were significantly reduced in the cells pretreated with both SPM-L and SPM-H for 24 h before $H_2O_2$ exposure (Figure 3), indicating a protective effect of SPM on the neuroblast cells.

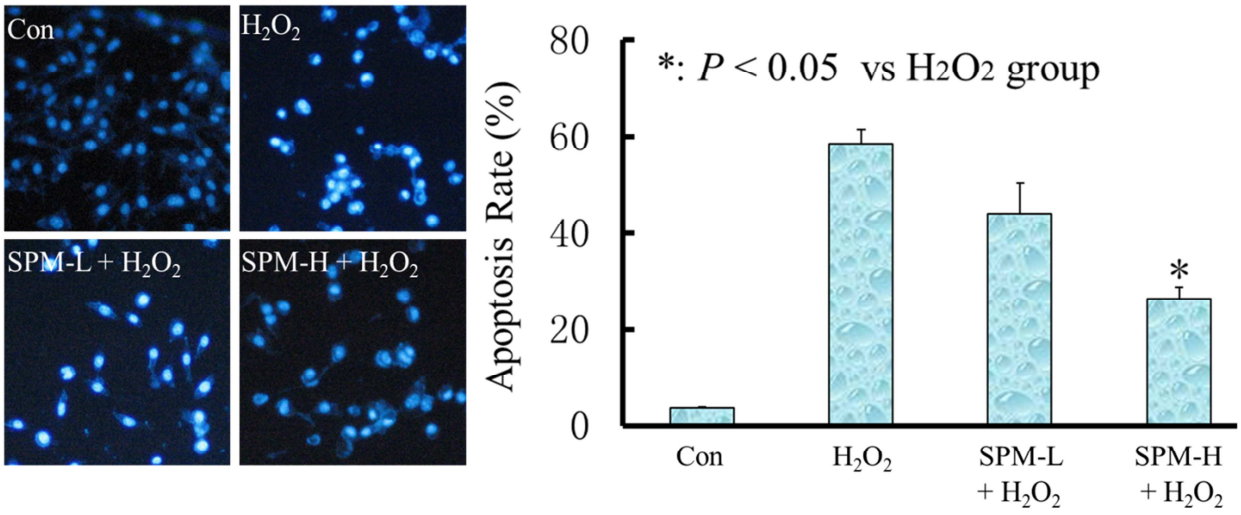


***Figure 3.*** *Representative photomicrographs of SH-SY5Y cell labelled with Hoechst 33342 (Panel A) after the indicated treatments. Fluorence intensity was calculated and mean (n=3) ± SD is presented (panel B). * p<0.05.*

Numerous studies have demonstrated that the loss of mitochondrial membrane potential is associated with apoptosis. Rh-123, a mitochondrial voltage-dependent dye, was employed to assess the changes in mitochondrial membrane potential. After exposure of SH-SY5Y cells to 1.54 mM $H_2O_2$ for 24 h, the fluorescence of Rh-123 decreased significantly (Figure 4), indicating the reduction of mitochondrial membrane potential. Pretreatment with SPM-L and SPM-H 24 h before $H_2O_2$ exposure resulted in a significant increase in mitochondrial membrane potential when compared with the $H_2O_2$ group (Figure 4).

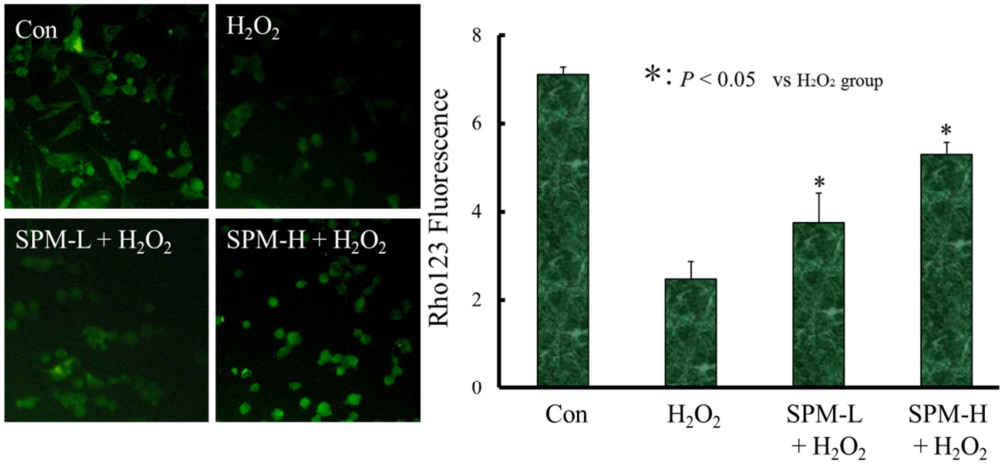


***Figure 4.*** *Representative photomicrographs of SH-SY5Y cells labelled with Rh-123. Panel A after 24 h exposure to indicated treatment. Fluorescence intensity was measured and mean (n=3) ± SD is presented (panel B). * p<0.05. ** p<0.01.*

### 3.3. The Effect of SPM on the Expression of PCNA and BDNA in $H_2O_2$-Exposed SH-SY5Y Cells

The effect of SPM on the expression of PCNA and BDNA in $H_2O_2$-exposed SH-SY5Y cells was investigated by western blot analysis and immunohistochemistry. Compared with the control group, $H_2O_2$ treatment significantly decreased the expression of both PCNA and BDNA in SH-SY5Y cells (Figure 5 and Figure 6). Pretreatment with SPM-L and SPM-H 24 h before $H_2O_2$ exposure restored much of the expression of PCNA and BDNA in $H_2O_2$-exposed SH-SY5Y cells (Figure 5 and Figure 6). These findings were also confirmed by western-blot analysis (Figure 7)

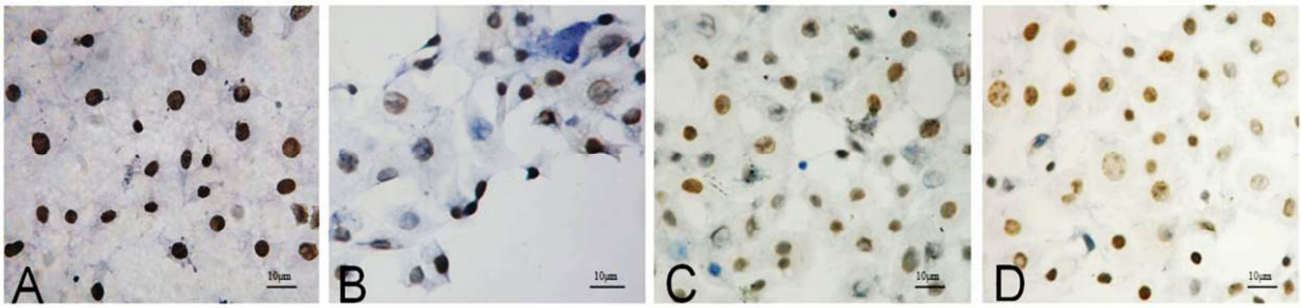


***Figure 5.*** *The expression of PCNA by immunochemistry in the SH-SY5Y cell (×200). A: Control; B: treated with $H_2O_2$; C: treated with SPM-L+$H_2O_2$; D: treated with SPM-H+$H_2O_2$.*

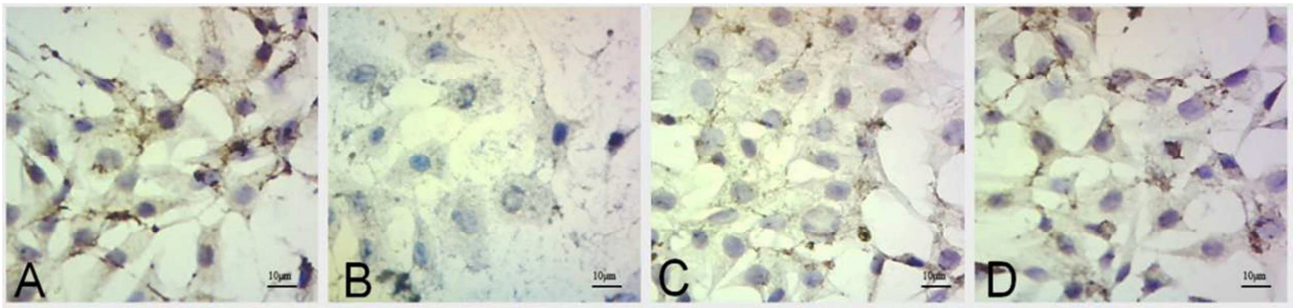


***Figure 6.*** *The expression of BDNF by immunochemistry in the SH - SY5Y cell (×200). A: Control; B: treated with $H_2O_2$; C: treated with SPM-L+$H_2O_2$; D: treated with SPM-H+$H_2O_2$.*

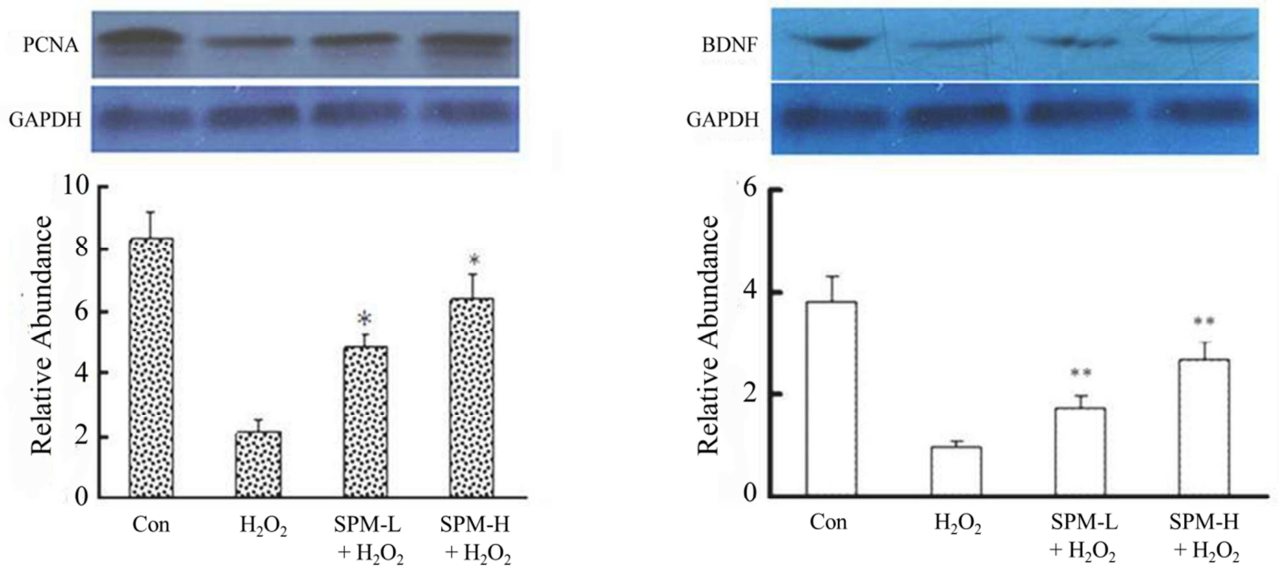


***Figure 7.*** *Expression of PCNA and BDNF by western-blot analysis in the SH - SY5Y cells. Positive bands were measured. * $p<0.05$. ** $p<0.01$ when compared to the cells with $H_2O_2$ treatment only.*

## 4. Discussion

Brain is a vital organ which is more susceptible to oxidative stress than any other organs due to its high metabolic rate and high content of polyunsaturated fatty acids. The imbalance between oxidants and antioxidants leads to disruption of redox signaling and oxidative stress [13]. The most common reactive oxygen species (ROS) include superoxide ($O^{2\cdot-}$), hydrogen peroxide ($H_2O_2$), peroxyl ($ROO^{\cdot}$) and reactive hydroxyl ($OH^{\cdot}$) radicals (Sies, 1997). These ROS play an important role in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases [14].

Oxidative stress has also been implicated in the development of dopaminergic neuron apoptosis, particularly in Parkinsonism [15]. In this investigation, we used dopaminergic SH-SY5Y human neurons as an in vitro model to assess the potential neuroprotective effect of SPM on oxidative stress by the exposure of $H_2O_2$. We found SPM had significant protective effects against H2O2 induced cell death, MMP dissipation and decrease in the expression of PCNA and BDNF. Pretreatment with SPM significantly decreased hydrogen peroxide-induced morphological change of nucleus and MMP dissipation. Immunohistochemistry and western-blot analysis demonstrated that SPM also prevents $H_2O_2$-related decrease in expression of PCNA and BDNF. These data suggest that SPM-provided neuron protection is possibly achieved through PCNA and BDNF up-regulations. PCNA is a DNA clamp and involved in many aspects of DNA replication-linked processes [16, 17]. BDNF, a member of the neurotrophic factor family, has been shown to promote survival and maintain proper function of neuronal population [18]. By examination of cell apoptosis, we found that SPM pretreatment suppressed $H_2O_2$-induced apoptosis and other forms of cell death. In addition, we demonstrated that this effect was associated with up-regulation of PCNA and BDNF. Taken together, these results for the first time provide evidence suggesting that SPM may exert its preventive effect of cell apoptosis on neuroblasts by increasing the expression of PCNA and BDNF. The results may provide insights for potential development of treatment to neurodegenerative diseases. To the best of our knowledge, this is the first study of the SPM on the protective effect on neurons. The results indicate that SPM has potential as a new drug candidate for neuro-degenerative diseases. Further studies are needed to identify the active ingredients from SPM for the development of more effective drug in the treatment of neurological disorders.

## 5. Conclusion

The snail polypeptide mixtures (SPM) could protect SH-SY5Y cells from apoptosis induced by $H_2O_2$, which holds promise as a novel drug for the treatment of neurodegenerative diseases.

## Acknowledgements

The authors thank all the participants and researchers in this study. This study was supported by the Application Technology Research and Development Foundation of Luoyang (1503001A).

## References

[1] Gao M, Yang Y, Lv M, Song W, Song Z. Oxidative stress and DNA damage in zebrafish liver due to hydroxyapatite nanoparticles-loaded cadmium. Chemosphere 2018; 202: 498-505.

[2] Ruan T, Li L, Lyu Y, Luo Q, Wu B. Effect of methionine deficiency on oxidative stress and apoptosis in the small intestine of broilers. Acta Vet Hung 2018; 66: 52-65.

[3] Ling Y, Gong Q, Xiong X, Sun L, Zhao W, Zhu W, Lu Y. Protective effect of madecassoside on $H_2O_2$-induced oxidative stress and autophagy activation in human melanocytes. Oncotarget 2017; 8: 51066-51075.

[4] Yoon JY, Kim DW, Kim EJ, Park BS, Yoon JU, Kim HJ, Park JH. Protective effects of remifentanil against $H_2O_2$-induced oxidative stress in human osteoblasts. J Dent Anesth Pain Med 2016; 16: 263-271.

[5] You HJ, Park JH, Pareja-Galeano H, Lucia A, Shin JI. Targeting MicroRNAs Involved in the BDNF Signaling Impairment in Neurodegenerative Diseases. Neuromolecular Med 2016; 18: 540-550.

[6] Machaalani R, Chen H. Brain derived neurotrophic factor (BDNF), its tyrosine kinase receptor B (TrkB) and nicotine. Neurotoxicology 2018; 65: 186-195.

[7] Jodeiri FM, Ghaedi K, Megraw TL, Curtiss J, Shirani FM, Vaziri P, Nasr-Esfahani MH. Does PGC1alpha/FNDC5/BDNF Elicit the Beneficial Effects of Exercise on Neurodegenerative Disorders? Neuromolecular Med 2016; 18: 1-15.

[8] Daigaku Y, Etheridge TJ, Nakazawa Y, Nakayama M, Watson AT, Miyabe I, Ogi T, et al. PCNA ubiquitylation ensures timely completion of unperturbed DNA replication in fission yeast. PLoS Genet 2017; 13: e1006789.

[9] Luo Y, Wang Q, Tian P, Jia Y. [Highly expressed CHAF1A and PCNA are positively associated with malignancy of cervical squamous cell carcinoma]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2017; 33: 1696-1701.

[10] Kashiwaba S, Kanao R, Masuda Y, Kusumoto-Matsuo R, Hanaoka F, Masutani C. USP7 Is a Suppressor of PCNA Ubiquitination and Oxidative-Stress-Induced Mutagenesis in Human Cells. Cell Rep 2015; 13: 2072-2080.

[11] Kowalska E, Bartnicki F, Fujisawa R, Bonarek P, Hermanowicz P, Tsurimoto T, Muszynska K, et al. Inhibition of DNA replication by an anti-PCNA aptamer/PCNA complex. Nucleic Acids Res 2018; 46: 25-41.

[12] Guan SW, Duan LX, Li YY, Wang BX, Zhou QL. A novel polypeptide from Cervus nippon Temminck proliferation of epidermal cells and NIH3T3 cell line. Acta Biochim Pol 2006; 53: 395-397.

[13] Saez-Freire M, Blanco-Gomez A, Castillo-Lluva S, Gomez-Vecino A, Galvis-Jimenez JM, Martin-Seisdedos C, Isidoro-Garcia M, et al. The biological age linked to oxidative stress modifies breast cancer aggressiveness. Free Radic Biol Med 2018; 120: 133-146.

[14] Niwa-Kawakita M, Ferhi O, Soilihi H, Le Bras M, Lallemand-Breitenbach V, de The H. PML is a ROS sensor activating p53 upon oxidative stress. J Exp Med 2017; 214: 3197-3206.

[15] Piri H, Haghdoost-Yazdi H, Fraidouni N, Dargahi T, Yaghoubidoust M, Azadmehr A. The Anti-Parkinsonism Effects of KATP Channel Blockade in the 6-Hydroxydopamine-Induced Animal Model: The Role of Oxidative Stress. Basic Clin Neurosci 2017; 8: 183-192.

[16] Buzovetsky O, Kwon Y, Pham NT, Kim C, Ira G, Sung P, Xiong Y. Role of the Pif1-PCNA Complex in Pol delta-Dependent Strand Displacement DNA Synthesis and Break-Induced Replication. Cell Rep 2017; 21: 1707-1714.

[17] Li F, Ball LG, Fan L, Hanna M, Xiao W. Sgs1 helicase is required for efficient PCNA monoubiquitination and translesion DNA synthesis in Saccharomyces cerevisiae. Curr Genet 2018; 64: 459-468.

[18] Zheng J, Sun J, Lu X, Zhao P, Li K, Li L. BDNF promotes the axonal regrowth after sciatic nerve crush through intrinsic neuronal capability upregulation and distal portion protection. Neurosci Lett 2016; 621: 1-8.