The Influence of Polygonatum King ilium on the Activity of Calcium-Regulating Enzymes and Calcium Ion Concentration in SH-SY5Y Cells Damaged by Aβ25-35 In Vitro

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Abstract: Objective To explore the effects of Polygonatum King ilium and Earthworm Formula (RPEWM) on the activity of calcium-regulating enzymes and free calcium ion (Ca^{2+}) concentration in SH-SY5Y cells with Aβ damage in vitro. Methods SH-SY5Y cells were induced to differentiate with retinoic acid, then damaged by Aβ to mimic Alzheimer's disease (AD) injury. RPEWM extract was added during the AD injury to protect the cells, and its effects on cell proliferation, apoptosis, and the activity of cellular Ca^{2+}-ATPase、Ca^{2+}-Mg^{2+}- ATPase ,as well as free calcium ion concentration were observed. Results RPEWM extract significantly protected cells from AD injury, resisted apoptosis, and promoted cell proliferation; it increased the activity of intracellular Ca^{2+}-ATPase、Ca^{2+}-Mg^{2+}- ATPase, reduced Ca^{2+} concentration, and showed a dose-dependent relationship. Conclusion RPEWM has a good protective effect on differentiated SH-SY5Y cells with $Aβ_{25-35}$damage and has a certain therapeutic effect on AD.

Keywords: Alzheimer's disease, Polygonatum King ilium and Earthworm Formula, SH-SY5Y cells; $Aβ_{25-35,}$ calcium ions, calcium ion-regulating enzymes.

1. Introduction

Polygonatum King ilium and Earthworm Formula is an original prescription developed by our research group based on traditional Chinese medicine theory. Preliminary research has found that this formula has a significant effect against senile dementia in rats and mice [1-2], and it provides good protection against $Aβ_{25-35}$damage in differentiated SH-SY5Y cells cultured in vitro [3]. On this basis, this study investigates the impact of this formula on the activity of calcium-regulating enzymes and the concentration of free calcium ions (Ca^{2+}) in the protective effect against $Aβ_{25-35}$ damage in differentiated SH-SY5Y cells, in order to explore its possible mechanism of action.

2. Materials and Methods

2.1. Experimental Drugs

RPEWM is composed of two traditional Chinese medicinal herbs, Polygonatum (Huang Jing) and Earthworm (Dilong), in a 1:1 ratio. Both Polygonatum and Earthworm were purchased from the outpatient pharmacy of the First Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine and were identified by the Department of Medicinal Materials and Specimen, Jiangxi University of Traditional Chinese Medicine. The Earthworm is classified as Guang Dilong, and the Polygonatum is classified as Polygala multiflora, both of which meet the national standards for clinical application of traditional Chinese medicine. Ginsenoside Rb1 standard was purchased from the China Institute for Food and Drug Administration (purity≥99.9%).Preparation of RPEWM concentrated extract [3]: 100g of Polygonatum and 100g of Earthworm were pulverized using an ultra-micro grinder. At room temperature, the powdered

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Polygonatum and Earthworm were mixed in a 1:1 ratio, 1000mL of distilled water was added for soaking, and stirred to ensure full immersion for 12hours; boiled and refluxed for 2hours, filtered, and the extract was collected. The residue from the coarse filtration was then re-added with 1000mL of distilled water (100℃), and boiled and refluxed again for 1hour, filtered, and the extract was collected; the two extracts were combined and centrifuged (2000g/min, 20min), the supernatant was collected, and the extract was concentrated by rotary evaporation to form 200mL of concentrated liquid (each 1mL is equivalent to 1g of crude drug) as the experimental RPEWM concentrated extract, which was filtered, sterilized by filtration, and stored at -20℃ for later use.

2.2. Experimental Reagents

Fetal bovine serum and DMEM/F12 medium are products of Gibco; Trypsin, all-trans retinoic acid, $A\beta_{25-35}$, MTT (thiazolyl blue tetrazolium bromide), Hoechst 33342, Fura-2/AM are products of Sigma; Calcium ion detection kit, Ca^2-ATPase, Ca^2-Mg^2-ATPase are products of Nanjing Jincheng Bioengineering Research Institute; All other reagents are domestically produced analytical grade reagents.

2.3. Experimental Equipment

The 752 spectrophotometer is a product of Shanghai Medical Analytical Instruments Factory, the multifunctional heat reflux traditional Chinese medicine extraction and concentration machine is a product of Shanghai Ding tai Evaporator Co., Ltd., and the fluorescence spectrophotometer and enzyme-linked immunosorbent assay (ELISA) reader are products of Thermo Fisher Scientific, USA. All others are common laboratory instruments.

2.4. SH-SY5Y Cell Culture [6]

Human neuroblastoma cells (SH-SY5Y cells) were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. The cells were cultured at a concentration of 5.0×10^4/mL in DMEM/F12 (1:1) medium containing 15% fetal bovine serum for experimental use.

2.5. Establishment of AD Cell Model

After 24hours of culture, SH-SY5Y cells were treated with all-trans retinoic acid at a final concentration of 1μmol/L for 3days to induce differentiation into neuronal cells. Then, 10μmol/L $A\beta_{25-35}$ was added to act on the neuronal cells for 48hours to establish the Alzheimer's disease (AD) cell model [7].

2.6. Cell Proliferation Assay (MTT Method)

Experimental Groups (the following experimental groups are the same): Cells were cultured in 96-well plates, induced to differentiate with retinoic acid, and then divided into normal control group, AD damage group, positive drug ginsenoside group, and three RPEWM extract concentration groups. The RPEWM extract groups were treated with $A\beta_{25-35}$ and simultaneously protected with RPEWM extract at concentrations of 30mg/L, 60mg/L, and 90mg/L (low, medium, and high doses), and then cultured for an additional 48hours before MTT assay was performed: The ginsenoside group was treated with $A\beta_{25-35}$ and simultaneously protected with ginsenoside Rb1 (final concentration of 50mg/L); The AD damage group was treated with $A\beta_{25-35}$; The normal control group was induced to differentiate with retinoic acid without $A\beta_{25-35}$ or drug protection. Each group had 10 replicate wells, and the experiment was repeated 10 times.

MTT Method: At the designated time point, add 20μL of MTT solution per well, and after 4hours, terminate the culture, discard the supernatant, add dimethyl sulfoxide (DMSO) (100μL per well), and agitate to dissolve the crystals. Measure the absorbance value [D570] at a wavelength of 570 nm using an automatic microplate reader, with higher D values indicating greater cell viability.

2.7. Hoechst 33342 Fluorescence Staining

At the designated time point, add a final concentration of 10μg/mL Hoechst 33342 to each well and incubate at 37°C for 30minutes, then add a final concentration of 1% paraformaldehyde to fix the cells in the dark for 30minutes. After centrifugation in the dark and discarding the supernatant, prepare cell smears

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and mount them onto glass slides with a coverslip using mounting medium. Observe the results under a fluorescence microscope. Randomly count 200 cells under high magnification and take photographs. Calculate the apoptosis rate: Apoptosis rate (%) = Number of apoptotic cells / Total number of counted cells × 100%.

2.8. Intracellular Ca²-ATPase, Ca²-Mg²-ATPase Activity, and Ca² Concentration Assay

At the designated time point, discard the cell culture medium for the detection of intracellular Ca²-ATPase, Ca²-Mg²-ATPase activity, and Ca² concentration. Enzyme activity assay: Add 150 μL of cell lysis solution to each group and incubate at 37°C for 30 minutes. According to the phosphate colorimetric method provided by the assay kit, add the detection reagents in sequence to detect the activities of Ca²-ATPase and Ca²-Mg²-ATPase, with detailed steps found in the assay kit instructions. Protein quantification is performed using the Lowry method. Ca² concentration detection: Add 150μL of cell lysis solution to each group, then add Fura-2/Am to a final concentration of 2μmol/L, and incubate at 37°C with constant temperature oscillation for 40minutes; then perform fluorescence measurement using a fluorescence spectrophotometer. The excitation wavelength for free Fura-2 is 340nm, and the excitation wavelength for Fura-2 bound to Ca² is 420nm. Measure the fluorescence intensity at these two wavelengths to determine their ratio (R). Calculate the content of [Ca²] i in each group using the formula [Ca²] i =K d(R – R min) / (R max - R), where K d is 224nmol/L, R min is the minimum fluorescence value (measured after the addition of EDTA to the suspension), and R max is the maximum fluorescence value (measured after the addition of 1% Triton-X100 to the suspension).

2.9. Statistical Analysis

Data were analyzed using the SPSS 11.5 statistical software package. Quantitative data are expressed as mean ± standard deviation (±SD) and were analyzed using the t-test. A difference was considered statistically significant at $P < 0.05$.

3. Results

3.1. Comparison of Ca²-ATPase, Ca²-Mg²-ATPase Activities, and Ca² Concentration Among Groups

Compared with the normal group, the damaged group showed a decrease in intracellular Ca²-ATPase and Ca²-Mg²-ATPase activities and an increase in Ca² concentration ($P < 0.01$); compared with the damaged group, the RPEWM extract groups at various concentrations exhibited increased intracellular Ca²-ATPase and Ca²-Mg²-ATPase activities ($P < 0.05$ or $P < 0.01$), and decreased Ca² concentration ($P < 0.05$ or $P < 0.01$), with a dose-dependent effect, and the differences were statistically significant. For details, see Table 1.

Table 1 Comparison of Ca^{2+}-ATPase、Ca^{2+}-Mg^{2+}-ATPaseActivities,Ca^{2+}Concentration Among Groups($\bar{X}$±S)

Group Categories Normal Group	Dosage (mg/L)	Ca^{2+}-ATPase U/mg	Ca^{2+}-Mg^{2+}-ATPase U/mg	[Ca^{2+}]i nmol/g
Damaged Group		6.14±0.26	5.82 ± 0.46	177.16±11.21
Ginsenoside Group		1.82±0.213)[4)]	1.39±0.11[3)]	752.89±45.25[3)]
RPEWM Group	50	4.26 ± 0.32[2)]	3.82±0.56[2)]	362.06±34.62[2)]
Group Categories	30	3.13±0.33[1)]	2.87±0.12[1)]	604.73±33.35[1)]
	60	4.14±0.48[2)]	3.53±0.45[2)]	447.47±21.23[2)]

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	90	5.06±0.37[2)]	4.62 ± 0.60[2)]	292.60±25.71[2)]
Compared with the Damaged Group, 1) P < 0.05, 2) P < 0.01; compared with the Normal Group, 3) P < 0.01.				

3.2. Comparison of Cell Proliferation and Apoptosis Rates Among Groups

The cell proliferation rate in the Damaged Group was significantly lower than that in the Normal Control Group (P<0.01); the cell proliferation rate in the RPEWM extract groups at all concentrations was higher than that in the Damaged Group (P<0.01), and the higher the dosage, the higher the cell proliferation rate, indicating significant cell protection. Under fluorescent microscopy, the cells in the Normal Control Group were uniform in size with a normal morphology, and the cytoplasm and nuclear chromatin exhibited a uniform blue fluorescence; in the Damaged Group, many cells showed condensed and intensely stained nuclear chromatin, enhanced fluorescence, and the nuclear chromatin was shrunken or fragmented into two or more pieces, indicating apoptotic cells; the number of apoptotic cells in the RPEWM extract groups at all concentrations was significantly reduced compared to the Damaged Group, and showed a dose-dependent relationship, with statistically significant differences compared to the Damaged Group (P<0.05 or P<0.01). For details, see Table 2.

Table 2 Comparison of Cell Proliferation and Apoptosis Rates Among Groups ($\bar{X}$±S) %

Group Categories	Dosage (mg/L)	Proliferation Rate	Apoptosis Rate
Normal Group		0.743±0.041	12.67 ± 3.61
Damaged Group		0.333±0.036[3)]	43.87±8.83[3)]
Ginsenoside Group	50	0.672±0.053[2)]	21.80±6.54[2)]
RPEWM Group	30	0.558±0.049[2)]	30.77 ± 5.11[1)]
	60	0.659±0.055[2)]	20.56±4.12[2)]
	90	0.690±0.062[2)]	18.59±5.44[2)]
Compared with the Damaged Group, 1) P < 0.05, 2) P < 0.01; compared with the Normal Group, 3) P < 0.01.			

4. Discussion

Dementia, also known as Alzheimer's disease (AD), was first reported by a German physician in 1906. It is an acquired, persistent intellectual disability characterized by progressive memory decline, cognitive impairment, and personality and emotional disorders, and is a brain degenerative disease [10]. Its etiology and pathogenesis are complex, with the main hypotheses currently being the cholinergic hypothesis, free radical hypothesis, calcium homeostasis imbalance hypothesis, genetic hypothesis, and apoptosis hypothesis. Therefore, clinical treatments for AD mainly focus on improving cholinergic neurotransmission, anti-free radical antioxidant, combating calcium overload in nerve cells, and neurotrophic and neuroprotective approaches [11-14].

Studies have confirmed that amyloid-beta (Aβ) is involved in AD induced by various causes, especially the main active fragment $A\beta_{25-35}$, which can lead to neurodegeneration, neurofibrillary tangles, and even neuronal death [15]. SH-SY5Y cells, derived from human neuroblastoma, share many bioinformatics characteristics with normal neurons and can differentiate into neuronal cells after induction with retinoic acid [7]. This study used SH-SY5Y cells induced to differentiate into neuronal cells by retinoic acid, then damaged by $A\beta_{25-35}$, to explore the possible mechanisms of action of RPEWM against AD.

Calcium ions have a significant impact on the proliferation, growth, differentiation, and death of somatic cells [16] and play an important role in the growth, development, synaptic connections, and signal

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transmission of nerve cells. With cellular aging, there is an energy metabolic disorder in maintaining membrane voltage, and the intracellular calcium ion concentration also changes accordingly [18]. Intracellular calcium ions are mainly maintained by the energy consumption of plasma membrane calcium channels and calcium pumps. When cells are damaged, it can cause in the mitochondrial oxidative phosphorylation process, leading to an energy metabolic disorder within the cell. The calcium pump cannot actively pump calcium out of the cell, causing calcium distribution disorders, leading to calcium balance system disorders. This is an important factor leading to neurodegenerative diseases such as Alzheimer's and Parkinson's syndromes [19]. Various free radicals produced by lipid peroxidation, especially reactive oxygen species, can also promote the opening of voltage-dependent calcium channels on the cell membrane, leading to calcium ion influx and calcium overload; conversely, calcium overload can further exacerbate the generation of various free radicals within the cell, and the two have a mutual influence, forming a vicious cycle. Excessive intracellular calcium ions are one of the initiating factors for inducing cell apoptosis, causing the expression of apoptosis-related genes, activating endonucleases, and leading to cell apoptosis [20].

5. Conclusion

The results of this study show that the proliferation rate of the cellular AD damage group was significantly lower than that of the normal control group, and cellular AD damage induced apoptosis. The RPEWM extract has a good protective effect against cellular AD damage and apoptosis, and its effect is dose-dependent. The detection of calcium-regulating enzyme activity and Ca^{2+} concentration found that $A\beta_{25-35}$can reduce the activity of Ca^{2+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase in SH-SY5Y cells, and increase the concentration of Ca^{2+}. The RPEWM extract can increase the activity of intracellular Ca^{2+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase, and reduce the concentration of Ca^{2+}, showing a dose-dependent correlation. This suggests that the anti-AD function of the RPEWM extract may be achieved by improving the activity of neuronal membrane Ca^{2+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase, restoring the normal function of various ions within the cell, especially the normal operation of Ca^{2+}, preventing calcium overload within the cell, and thus playing a role in anti-free radical, antioxidant, and anti-apoptotic effects. The specific mechanism awaits further research.

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