Cinnamaldehyde promotes mitochondrial function and reduces Aβ toxicity in neural cells

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Abstract: Cinnamon and its major active component, cinnamaldehyde, have been shown to be neuroprotective in models of Alzheimer's disease (AD). To further investigate the mechanism of cinnamaldehyde, we investigated the effects of cinnamaldehyde focusing on mitochondrial function in SH-SY5Y neural cells. The results demonstrated that cinnamaldehyde could enhance neural cell viability with or without increased Aβ levels. Cinnamaldehyde facilitated the maintenance of normal mitochondrial morphology, preserved the mitochondrial membrane potential ($\Delta\Psi_m$), and reduced production of reactive oxygen species (ROS). Cinnamaldehyde also decreased the expression of dynamin-related protein 1 (Drp1), a protein critically involved in mitochondrial dynamics. In addition, cinnamaldehyde inhibited Aβ oligomerization, but it had no effects on Tau phosphorylation. In overall, cinnamaldehyde promoted mitochondrial function and inhibited Aβ toxicity, and these two properties may both contribute to the neuroprotective effect. These results suggest that cinnamaldehyde could be a potential nutriceutical in the prevention and even therapeutic treatment of AD as well as other aging-related metabolic syndromes.
Keywords: Alzheimer's disease, Aβ oligomers, Mitochondria, Cinnamaldehyde

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1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder, with progressive memory impairment and other dysfunctions, especially in the late stages of the disease[1]. According to the Alzheimer's association, AD is the fifth major factor of death in elderly patients[2]. The amyloid hypothesis (Aβ aggregation, along with formation of neurofibrillary tangles (NFT) containing hyperphosphorylated Tau) has been well accepted as one of the key AD pathogenesis mechanisms[3,4]. Additional proposed mechanisms, including neurovascular dysfunction, cell-cycle abnormalities, inflammatory processes, oxidative stress, and mitochondrial dysfunction have been studied as well[5–7]. Unfortunately, despite decades of intensive investigation, no disease modifying therapies (methods designed to reduce the brain amyloid burden) have been successful[8] and novel strategies are needed for AD treatment[9].

One of the under-investigated areas for AD therapeutic agents is natural products, especially traditional Chinese medicines (TCM)[10]. Early studies have indicated that cinnamon, a commonly used spicy food flavouring and a TCM agent, may be useful[11]. Cinnamon exhibits a variety of biological and pharmacological effects including, antioxidant[12] and anti-inflammatory[13–15], antimicrobial[16–18], and anticancer activities[19]. Cinnamon is also shown to have insulin-potentiating properties, and it may be involved in the alleviation of the signs and symptoms of diabetes and cardiovascular diseases related to insulin resistance and metabolic syndrome[11,20–27]. Interestingly, oral administration of cinnamon extracts effectively inhibits formation of toxic oligomeric Aβ species, reduces Aβ plaques, and improves cognitive behavior in AD animal models[28,29]; moreover, cinnamaldehyde,

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a major bioactive component in cinnamon, appears to protect Tau from oxidation-induced modifications and to inhibit tau aggregation[29]. Nonetheless, the mechanism by which cinnamon or cinnamaldehyde attenuates Aβ aggregation or tau species-mediated toxicity remains unclear.

Considering the relationship between type 2 diabetes and AD[30,31], we postulated that cinnamon or cinnamaldehyde may exert its affects by regulating the mitochondrial function of neural cells in addition to inhibiting Aβ oligomerization and Tau modification. In fact, the mitochondrial cascade hypothesis of AD has emphasized that mitochondrial dysfunction could be the main cause of Aβ plaques and NFTs in sporadic late-onset AD[32]. In this study, we investigated and compared the effects and mechanisms of cinnamaldehyde on neural cells (SH-SY5Y) and cells overexpressing Aβ, focusing on the effects of cinnamaldehyde on mitochondrial function.

2. Materials and methods

2.1. Materials

Cinnamaldehyde was obtained from Beijing Ouhe Technology Co., Ltd (China). Anti-β-amyloid 1–16 antibody (6E10) was obtained from Biolegend (USA). Anti-Tau (phospho T231) antibody and anti-DRP1 antibody were obtained from Abcam (UK). Rabbit polyclonal antibody against GAPDH was obtained from Bioeasy (China). Fetal bovine serum (FBS) was obtained from Cellgro (USA). DMEM culture medium and phosphate buffered saline (PBS) were obtained from Beijing North TZ-Biotech Develop., Co. Ltd (China). Human β-amyloid (1-42) was obtained from ChinaPeptides (China). CellTiter 96® AQueous One Solution Cell Proliferation Assay was obtained from Promaga Co. (USA). MitoTracker® Mitochondrion-Selective Probes were obtained from Invitrogen Co. (USA). Reactive Oxygen Species Assay Kit was obtained from Beyotime (China). Mitochondrial Membrane Potential Assay Kit with JC-1 was obtained from Bridgen (China). Other reagents were of analytical grade.

2.2. Cell culture and treatment

Three human neuroblastoma cell lines (SH-SY5Y-neo, SH-SY5Y-APPwt, and SH-SY5Y-APPswe) were obtained from the Institute of Biophysics, Chinese Academy of Sciences[33]. SH-SY5Y-APPwt cells express wild type Aβ precursor protein (APP). SH-SY5Y-APPswe cells express APP with the Swedish mutation. SH-SY5Y-neo are blank cells transfected with an empty vector. SH-SY5Y cells were grown in DMEM medium supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin in an incubator containing 5% CO_2 at 37 °C. For maintaining the stably transfected SH-SY5Y cells expressing APP, G418 (100 μg/mL) was included in the culture media.

2.3. MTS assay for cell viability

Cell viability was measured by quantitative colorimetric MTS assay, which serves as an indication of the mitochondrial activity of living cells[34]. Briefly, the SH-SY5Y cells were cultured on 96-well plates until 70%–80% confluence. Then the cells were then incubated in DMEM medium supplemented with 10% FBS with various concentrations of cinnamaldehyde or other indicated reagents for 36 h or the indicated time periods. Then, the medium was removed and fresh medium containing 100 μL/mL MTS was added to each well and incubated for 3 h at 37 °C. Finally, the absorbance at 490 nm of each aliquot was determined with a microplate reader (Thermo Scientific, USA).

2.4. Western blotting assay

The SH-SY5Y cells were seeded on 6-well plates until 70%–80% confluent. After treatment with 100 μM cinnamaldehyde or the indicated reagents for 36 h or the indicated time periods, the cells were washed twice in ice-cold PBS and lysed on ice with RIPA lysis buffer (Beyotime, China) supplemented with 1× protease inhibitor cocktail (Applygen, China) and 1× phosphatase inhibitors (Leagene, China). The cell lysates were centrifuged at 4 °C for 20 min at 12 000 ×g. Then the supernatant

was collected and the total protein levels were determined with a bicinchoninic acid assay kit (BCA, Pierce) according to the manufacturer's instructions. The samples (containing 40 μg of total protein) were separated on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF, Millipore). The membrane was blocked in 5% BSA-TBST for 1 h and incubated with desired primary antibodies (β-Amyloid 1-16, 1:2000; Tau (phospho T231), 1:4000; DRP1, 1:1000; GAPDH, 1:4000) overnight at 4 ºC, followed by incubation with HRP-conjugated secondary antibodies (1:5000, Easybio) for 1 h at room temperature. After washing, the protein bands were visualized with an ECL Western blotting substrate kit (Kang Biquan Co., Ltd., China) on an X-ray film (Easybio, China). The protein bands were analyzed and quantified by ImageJ (National Institutes of Health, USA). The strip intensity was expressed as the ratio of the target protein to GAPDH (i.e. Drp1/GAPDH) and normalized to the control samples.

2.5. *In vitro* Aβ oligmerization/aggregation assay

The effect of cinnamaldehyde on Aβ aggregation was analyzed using a modified method of Craig S. Atwood et al[35]. Briefly, aliquots of 0.5 μM Aβ1-42 in 150 mM NaCl and 20 mM HEPES (pH 7.4) containing 20 μM of $CuSO_4$ were incubated in the presence and absence of cinnamaldehyde (100 μM) or the indicated reagents at 37 °C for 60 min. Then the mixtures were separated by 10% SDS-PAGE and electrically transferred onto a polyvinylidene difluoride membrane (PVDF, Millipore). The membrane was blocked in 5% BSA-TBST and incubated with primary antibodies to β-Amyloid 1-16 (1:2000, Biolegend) at 4 ºC overnight, followed by incubation with HRP-conjugated secondary antibodies (1:5000, Easybio) for 1 h at room temperature. After washing, the protein bands were visualized with an ECL Western blotting substrate kit (Kang Biquan Co., Ltd., China) on an X-ray film (Easybio, China). The protein bands were analyzed and quantified by ImageJ (National Institutes of Health, USA).

2.6. Measurement of intracellular ROS

The SH-SY5Y cells were grown in 6-well culture dishes. After drug treatment, the cells were collected and incubated with 10 μM of DCFH-DA at 37 °C for 20 min. Then cells were washed three times with FBS-free DMEM medium. The fluorescence intensity of DCF was determined using flow cytometry (Beckman Coulter, USA) with the excitation wavelength at 488 nm and the emission wavelength at 525 nm.

2.7. Mitochondrial labeling and confocal microscopy

The SH-SY5Y cells were grown on 35-mm^2 confocal dishes (Axygen, USA). After drug treatment, the cells were incubated with 50 nM MitoTracker Red CMXRos for 10 min at 37 °C. Then the cells were washed three times with FBS-free DMEM medium and imaged on a confocal laser scanning microscope (Nikon, Japan) with an excitation wavelength at 579 nm and an emission wavelength at 599 nm.

2.8. Assessment of mitochondrial membrane potential

Fluorescent probe JC-1 (Bridgen) was used to detect the mitochondrial membrane potential ($\Delta\Psi_m$) of the SH-SY5Y cells upon drug treatment[36]. Briefly, the SH-SY5Y cells were grown in 6-well culture dishes. After drug treatment, the medium was removed and fresh medium containing JC-1 (1×) was added and left for 20 min at 37 °C. Then the cells were washed twice with JC-1 staining buffer (1×) and imaged by fluorescence microscopy (Olympus, Japan) with an excitation wavelength of 550 nm and an emission wavelength 600 nm (the red fluorescence channel) or an excitation wavelength of 485 nm and an emission wavelength of 535 nm (green fluorescence channel).

2.9. Statistical analysis

The data were expressed as mean±SD. Differences in data between groups were compared with one-way ANOVA test by GraphPad Prism 5.0. The threshold

for statistical significance was set at $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, respectively.

3. Results

3.1. The effects of cinnamaldehyde on cell viability of SH-SY5Y neural cells

As shown in Figure 1, cinnamaldehyde increased the viability of all three neural cells in the tested concentration range of 40–120 μM with or without APP over-expression. For normal SH-SY5Y cells, cinnamaldehyde improved viability by ~50% (n = 3, $P<0.05$). For cells over-expressing Aβ precursor protein (i.e. SH-SY5Y-APPwt and APPswe), cinnamaldehyde increased the cell viability by 10%–20% (n = 3, $P<0.05$). However, cinnamaldehyde decreased the cell viability at concentrations over 160 μM. Therefore, the optimal concentration (100 μM) of cinnamaldehyde was used in the following experiments.

3.2. Effects of cinnamaldehyde on intracellular ROS levels

The effects of 100 μM cinnamaldehyde on intracellular ROS levels of the three neural cells are shown in Figure 2. Cinnamaldehyde decreased the intracellular ROS levels by ~20% (n = 3, $P<0.05$) in all three types of cells.

3.3. Effects of cinnamaldehyde on mitochondrial $\Delta\Psi_m$

The mitochondrial membrane potential ($\Delta\Psi_m$) of the three neural cells treated with 100 μM of cinnamaldehyde was measured with a cell-based JC-1 assay. Changes in $\Delta\Psi_m$ were identified by changes in the red to green fluorescence ratio. As shown in Figure 3, cinnamaldehyde did not affect the $\Delta\Psi_m$ of SH-SY5Y-neo cells. In contrast, cinnamaldehyde appeared to increase the $\Delta\Psi_m$ by 30%–50% in SH-SY5Y-APPwt and SH-SY5Y-APPswe cells, however, the difference was not statistically significant.

3.4. Effects of cinnamaldehyde on mitochondrial morphology

One of the major targets of Aβ-induced toxicity is mitochondrial dysfunction. Overexpression of APP in neural cells induced fission of mitochondria, generating a large amount of round swollen mitochondria[37]. As shown in Figure 4, confocal images of SH-SY5Y cells upon treatment with MitoTracker revealed several observations including: 1) most mitochondria of SH-SY5Y-neo cells appeared thread-like (Fig. 4A) and the cinnamaldehyde treatment did not affect it significantly; 2) SH-SY5Y-wt cells gave mixture of both thread-like and rounded mitochondria (Fig. 4B), and cinnamaldehyde treatment almost completely restored the mitochondrial

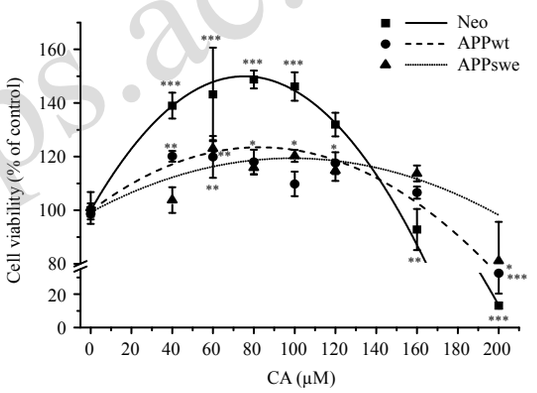

Figure 1. Effects of different concentrations of cinnamaldehyde on cell viability. SH-SY5Y-neo, SH-SY5Y-APPwt and APPswe cells were incubated with indicated concentrations of cinnamaldehyde for 36 h at 37 °C. $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$ compared with the untreated cells. Results are mean±SD, n = 3. Statistical analysis was done by one-way ANOVA test.

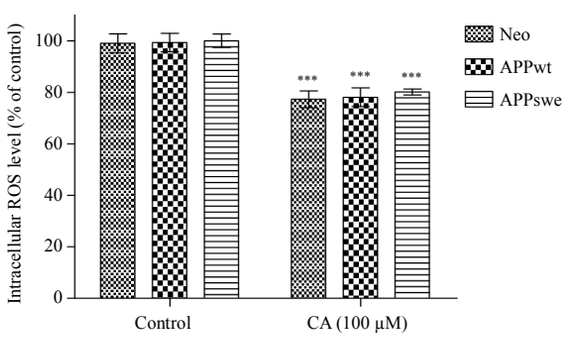

Figure 2. Effects of cinnamaldehyde on intracellular ROS. Cells were treated with 100 μM cinnamaldehyde for 36 h at 37 °C. $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$ compared with the untreated cells. Results are mean±SD, n = 3. Statistical analysis was done by one-way ANOVA test.

shape; 3) SH-SY5Y-swe cells exhibited mostly swollen round shape mitochondria (Fig. 4C), and cinnamaldehyde treatment returned most of the mitochondria to a normal thread-like morphology.

3.5. Effects of cinnamaldehyde on mitochondrial Drp1 expression

Drp1 is a key protein that controls mitochondrial fission and mitochondrial morphology[38]. As shown in Figure 5, in all three neural cells, cinnamaldehyde significantly down-regulated the levels of Drp1, indicating that suppression of mitochondrial fission might be involved in its therapeutic mechanism.

3.6. Effects of cinnamaldehyde on Tau phosphorylation

Hyperphosphorylation of Tau is a key event in Tau aggregation and subsequent formation of neurofibrillary

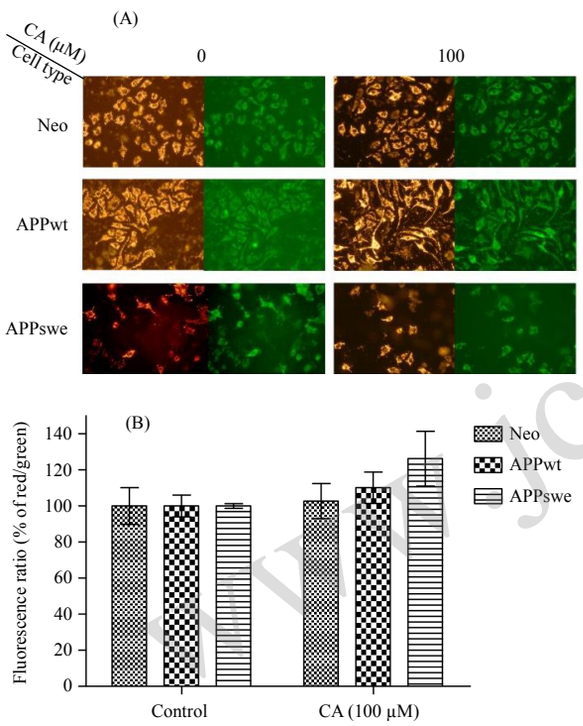

Figure 3. Effect of cinnamaldehyde on the mitochondrial membrane potential (ΔΨm) in SH-SY5Y-neo, APPwt and APPswe cells (40×). (A) The fluorescence images of the neural cells upon treatment with cinnamaldehyde; (B) the ratio of red: green fluorescence of the cells. The $\Delta\Psi_m$ was assayed with the JC-1 staining as described in Materials and method. Results are mean±SD, n = 3. Statistical analysis was performed with a one-way ANOVA test.

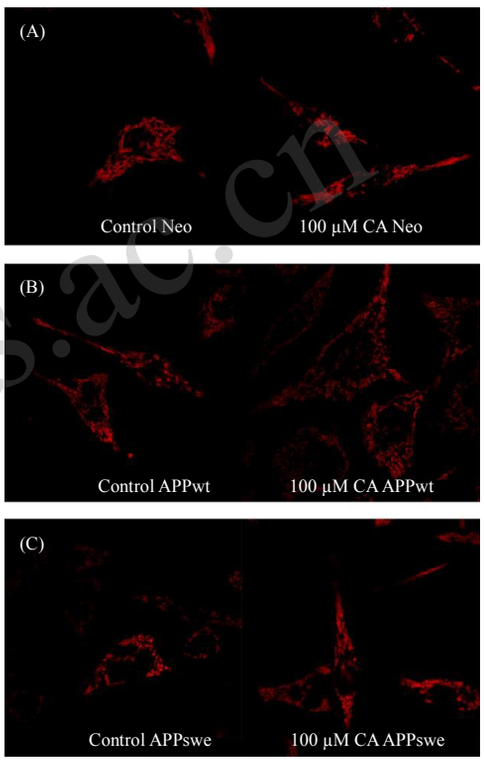

Figure 4. The morphology of mitochondria of neural cells upon treatment with 100 μM of cinnamaldehyde (100×). (A) SH-SY5Y-neo cells; (B) SH-SY5Y-wt cells; (C) SH-SY5Y-swe cells. Mitochondria were labeled with MitoTracker Red CMXRos and observed under a confocal laser scanning microscope.

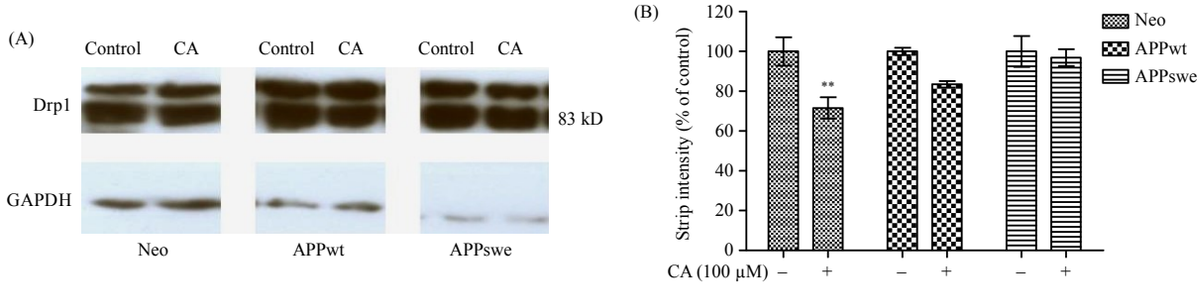

Figure 5. Western blotting analysis of Drp1 protein in SH-SY5Y neural cells. (A) Western blotting results of three cell lines upon 100 μM cinnamaldehyde treatments; (B) quantification of the western blotting results in (A). $^{***}P$<0.001, $^{**}P$<0.01 and $^{*}P$<0.05 compared with the untreated cells. Results are mean±SD; n = 3. Statistical analysis was done by one-way ANOVA test.

tangles (NFT)[39,40]. As shown in Figure 6, cinnamaldehyde had no significant effect in SH-SY5Y-wt or SH-SY5Y-swe cells, but it significantly reduced the level of p-Tau in SH-SY5Y-neo cells.

3.7. Effects of cinnamaldehyde on Aβ oligomerization

The Aβ oligomers are the primary toxic Aβ forms mediating neurodegeneration in AD[41]. Certain metal ions, especially Cu^{2+}, have been proposed as a key trigger. The effect of cinnamaldehyde on Cu^{2+}-induced Aβ oligomerization *in vitro* were tested herein and the results (Fig. 7) indicated that $Aβ_{1-42}$ by itself tended to form tetramers (the band at ~15 kDa) and the amount of Aβ tetramers increased significantly upon the addition of Cu^{2+}. Cinnamaldehyde substantially decreased the amount of Aβ tetramers (n = 3, P<0.05).

The effect of cinnamaldehyde on Aβ oligomerization in SH-SY5Y-Neo or SH-SY5Y-wt cells were examined as well and the results (Fig. 8) showed that cinnamaldehyde decreased Aβ oligomers with statistical significance (n = 3, P<0.05).

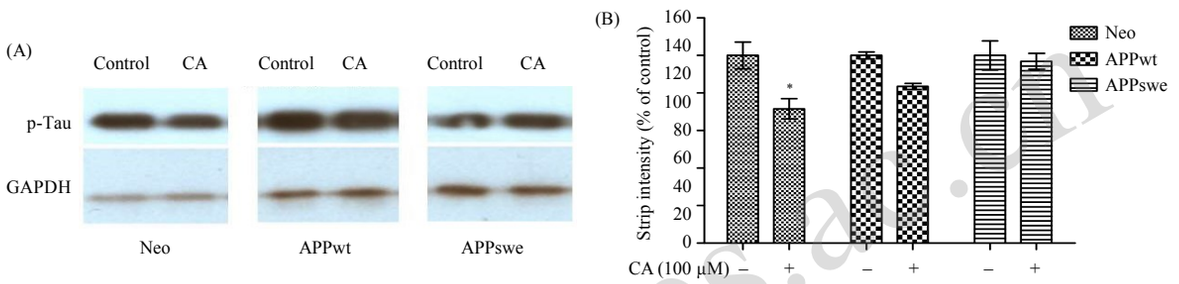

Figure 6. Western blotting analysis for p-Tau levels in SH-SY5Y neural cells. (A) Western blotting results of three cell lines upon 100 μM cinnamaldehyde treatments; (B) quantification of the western blotting results in (A). $^{***}P$<0.001, $^{**}P$<0.01 and $^{*}P$<0.05 compared with the untreated cells. Results are mean±SD, n = 3. Statistical analysis was done by one-way ANOVA test.

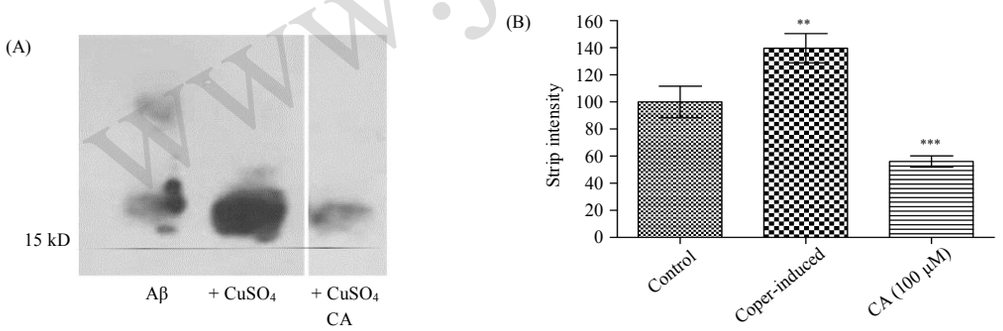

Figure 7. Western blot analysis for Aβ aggregation induced by copper. (A) Western blotting results; (B) quantification of the results in (A). $^{***}P$<0.001, $^{**}P$<0.01 and $^{*}P$<0.05 compared with the copper-induced group. Results are mean±SD, n = 3. Statistical analysis was done by one-way ANOVA test.

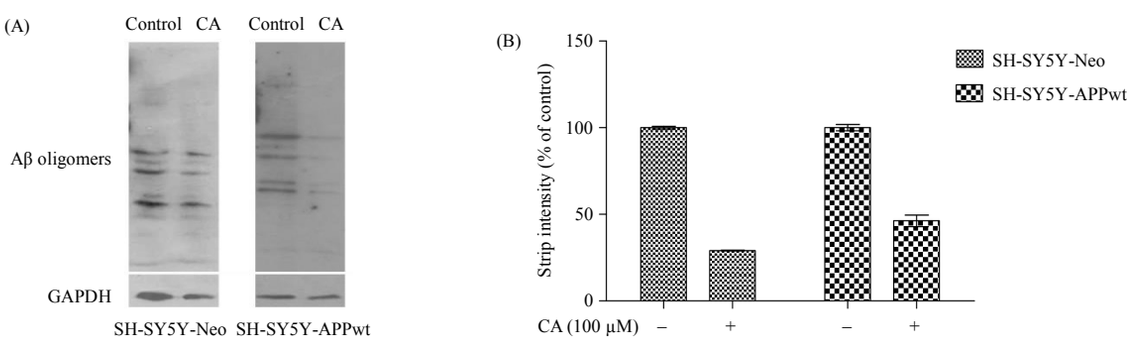

Figure 8. Western blotting for Aβ oligomers in SH-SY5Y-wt AD cell model. (A) Western blotting results; (B) quantification of the results in (A). Results are mean±SD, n = 3. Statistical analysis was done by one-way ANOVA test.

4. Discussion

The primary discovery of these studies was that cinnamaldehyde, one of the major active components of cinnamon, enhanced mitochondrial function, likely by regulating Drp1 expression, and neuronal survival both with and without APP overexpression.

Our results demonstrated that cinnamaldehyde enhances mitochondrial function because cinnamaldehyde promoted mitochondrial activity (Fig. 1), reduced ROS in three cell lines (Fig. 2), maintained mitochondrial membrane potential ($\Delta\Psi_m$, Fig. 3), and most importantly, restored the normal morphology of mitochondria in cells producing Aβ peptides (Fig. 4). It has been suggested that in a large number of aging and neurodegenerative diseases including AD, impairment of mitochondrial dynamics involves excessive mitochondrial fission[37,38], resulting in mitochondrial structural changes and dysfunction. Therefore, inhibitors of mitochondrial fission have recently been regarded as a novel therapeutic strategy for treating AD[37,38]. Overall, the results suggested that the fundamental function of cinnamaldehyde is to promote the general health of cells, not necessarily specifically against Aβ toxicity.

It is notable that cinnamaldehyde down-regulated Drp1 protein levels in all three cell lines (Fig. 5). Given that Drp1 is critically involved in mitochondrial fission and mitophagy[38], it is plausible that cinnamaldehyde at optimal concentrations (Fig. 1) can tip the balance of mitochondrial dynamics, favoring cell survival. However, mitochondrial fission and fusion involves a variety of enzymes (e.g. Drp1, Fis1, Mfn1, Mfn2, and Opa1), and the mechanism by which cinnamaldehyde maintains healthy mitochondrial dynamics remains to be further investigated in future work.

The mitochondria are key sites of cellular energy production and glucose/lipid metabolism[42,43], and the effects of cinnamaldehyde on mitochondria agree well with the activity of cinnamon in the alleviation of the signs and symptoms of diabetes and cardiovascular diseases[44]. It was reported that cinnamon extract can potentiate insulin activity and ameliorate insulin resistance and metabolic syndrome[44].

We also observed that cinnamaldehyde could reduce Aβ toxicity by inhibiting Aβ oligomerization (Fig. 7 and Fig. 8), which is in good agreement with previous studies[45]. However, we believe that the effect is likely secondary to preservation of mitochondrial function. Additionally, in contrast to previous studies[29], we did not observe an obvious effect of cinnamaldehyde on the level of Tau phosphorylation, a key event associated with formation of NFT, when APP was overexpressed (Fig. 6). This could be due to many factors, such as the fact that there was no apparent NFT formation anyway in our cell cultures.

In conclusion, our present work suggests that cinnamaldehyde, an active ingredient in the human diet, could maintain normal mitochondrial dynamics, promote mitochondria activity, and inhibit Aβ oligomerization, thereby reducing Aβ toxicity and neural cell damage. Our results support the use of cinnamaldehyde in prevention and therapeutic treatment of AD and other aging-related metabolic syndromes.

Acknowledgements

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肉桂醛促进神经细胞线粒体功能并抑制Aβ毒性

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摘要: 一些研究表明肉桂及其主要活性成分肉桂醛具有抗阿尔兹海默症(AD)活性。为了阐明肉桂醛的作用机制、促进抗AD药物开发, 本文研究了肉桂醛对SH-SY5Y神经细胞特别是线粒体的作用。实验结果表明, 肉桂醛能够促进神经细胞在有和无β-淀粉状蛋白(Aβ)负载下的细胞活力, 其作用机制包括: 保护和恢复Aβ损伤的正常线粒体形态、维持线粒体膜电位和降低ROS的产生。肉桂醛导致Drp1蛋白表达可能与其阻止Aβ诱导线粒体裂解有关。此外, 肉桂醛也能抑制Aβ的寡聚化, 降低其细胞毒性。但肉桂醛对SH-SY5Y神经细胞Tau蛋白的磷酸化却没有明显作用。总之, 肉桂醛可促进神经细胞线粒体功能并抑制Aβ毒性, 这两个机制在神经保护方面互相关联。本文结果提示肉桂醛可以作为保健药物用于AD和其他衰老性代谢疾病的防治。

关键词: 阿尔茨海默症; β样淀粉蛋白; 线粒体; 肉桂醛