RESEARCH ARTICLE

Enzyme-digested *Colla Corii Asini* (E'jiao) prevents hydrogen peroxide-induced cell death and accelerates amyloid beta clearance in neuronal-like PC12 cells

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Abstract

As an aging-associated degenerative disease, Alzheimer's disease is characterized by the deposition of amyloid beta (Aβ), oxidative stress, inflammation, dysfunction and loss of cholinergic neurons. *Colla Corii Asini* (CCA) is a traditional Chinese medicine which has been used for feebleness-related diseases and anti-aging. CCA might delay aging-induced degenerative changes in neurons. In the present study, we evaluated antioxidant activity, cytoprotective effects, and Aβ removability of enzyme-digested *Colla Corii Asini* (CCAD). Oxygen radical absorbance capacity (ORAC) activity assay showed that, as compared to gelatins from the skin of porcine, bovine and cold water fish, CCA exhibited the highest ORAC activity. The ORAC activity of CCA and CCAD was increased gradually by the length of time in storage. Ultrastructure analysis by scanning electron microscopy showed that among CCA manufactured in 2008, 2013, 2017 and gelatin from cold water fish skin, CCA manufactured in 2008 presented the smoothest surface structure. We further tested the protective effects of CCAD (manufactured in 2008) and enzyme-digested gelatin from cold water fish skin (FGD) on hydrogen peroxide (H_2O_2)-induced cell death in nerve growth factor-differentiated neuronal-like PC12 cells. Presto blue assay showed that both FGD and CCAD at 0.5 mg/mL increased cell viability in H_2O_2-treated neuronal-like PC12 cells. The protection of CCAD was significantly superior to that of FGD. Acetylcholinesterase (AchE) assay showed that both FGD and CCAD inhibited AchE activity in nerve growth factor-differentiated neuronal-like PC12 cells to 89.1% and 74.5% of that in non-treated cells, respectively. The data suggest that CCAD might be able to increase the neurotransmitter acetylcholine. Although CCAD inhibited AchE activity in neuronal-like PC12 cells, CCAD prevented H_2O_2-induced abnormal deterioration of AchE. ELISA and neprilysin activity assay results indicated that CCAD reduced amyloid beta accumulation and increased neprilysin activity in $A\beta_{1-42}$-treated neuronal-like PC12 cells, suggesting that CCAD can enhance Aβ clearance. Our results suggest that CCA might be useful for preventing and treating Alzheimer's disease.

Key Words: *acetylcholinesterase activity; Alzheimer's disease; amyloid beta clearance; antioxidant; Colla Corii Asini; collagen; neuroprotection; PC12 cells*

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder. Deposition of amyloid beta (Aβ) is considered as the primary causative mediator of the pathology of AD (Hardy and Higgins, 1992). Abnormal accumulation of Aβ results in a series of brain lesions, including neurofibrillary tangles, oxidative stress, neural inflammation, and functional neuron loss (Ricciarelli and Fedele, 2017). The imbalance of the production and clearance of Aβ causes the formation of Aβ plaques. Evidences indicate that, in the majority of cases of AD, Aβ clearance is impaired (Wildsmith et al., 2013) due to the dysfunction of Aβ-degrading enzymes, such as neprilysin (Hellström-Lindahl at al., 2008; Baranello et al., 2015). However, despite extensive research and drug development aiming at the prevention and removal of Aβ plaques, there are no successful outcomes in clinical trials (Cummings et al., 2016). Since inflammation (Kinney et al., 2018) and oxidative stress (Wang et al., 2014b) are the two main contributors for functional neuron death in AD brains, the treatment that can delay the progress of AD should be able to 1) offer neuroprotection against oxidative stress and inflammation-induced neuron loss; 2) accelerate Aβ clearance; 3) restore functional neurons, especially cholinergic neurons (cholinergic neurons are severely lost in AD patients) (Ferreira-Vieira et al., 2016).

Colla Corii Asini (CCA) is a traditional Chinese medicine made of the extract from donkey skin. CCA has been used in Asia to treat hemostasis, miscarriage, and feebleness-related diseases for more than 2500 years (Xiao et al., 2019a). CCA is a mixture of peptides and proteins produced by partial

hydrolysis of collagen (Wang et al., 2014a). As a biomaterial, collagen has diverse medical applications, such as bone and cartilage reconstruction, wound dressing, and tissue engineering. Recently, evidences show that collagen might have effects on neuroprotection and neuroregeneration (Ucar and Humpel, 2018). In the present study, we used CCA to treat a well-known neuronal cell line PC12 to check its neuroprotective effects and potential therapeutic applications for AD patients.

Materials and Methods

Reagents

CCAs manufactured in different years (2017, 2013 and 2008) were provided by Dong-E-E-Jiao Co. Ltd., China. Gelatin from cold water fish skin (G7041), gelatin from porcine skin (G1890), gelatin from bovine skin (G9391), nerve growth factor (NGF; SRP3015), acetylcholinesterase (AchE) from *Electrophorus electricus* (C3389), acetylthiocholine iodide (A5751) and 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (96669) were purchased from Sigma-Aldrich, Tokyo, Japan. Dulbecco's modified Eagle's medium (DMEM) (043-30085), donepezil hydrochloride (045-32321) and chemicals other than mentioned in the main text were purchased from FUJIFILIM Wako Pure Chemical Co., Osaka, Japan.

Preparation of enzyme-digested gelatins

CCAs were melted in double distilled water (DDW) at 50 mg/mL. CCA solutions (CCA 2017, CCA 2013 and CCA 2008) were digested *in vitro* according to the modified method (Vermeirssen et al., 2003). To simulate the digestion in the stomach and the small intestine, gastric juice, bile and duodenal juice were made (**Table 1**). CCA solutions were first reacted with gastric juice at a volumetric rate of 1 (gastric juice): 125 (CCA solution) at 37°C for 30 minutes and then reacted with bile (1 bile: 112 CCA solution) and duodenal juice (1 duodenal juice: 37 CCA solution) at 37°C for another 30 minutes. The gastrointestinal digests were filtered through 0.22 μm filters and kept at –80°C until use.

Oxygen radical absorbance capacity assay

Oxygen radical absorbance capacity (ORAC) assay kit was purchased from Cell Biolabs Inc., USA. The experiments were performed according to the manufacturer's protocol with some modifications. The water-soluble vitamin E analog Trolox™ was used as the antioxidant standard. Briefly, samples (25 μL) were first added into the wells of a microplate (black 96-well plates, Nunc™ black microwell, Thermo Fisher, Scientific, Tokyo, Japan). Fluorescein (150 μL; 70 nM final concentration) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) (25 μL; 12 mM final concentration) solutions were quickly mixed in a pipetting reservoir and added to the wells of samples rapidly by using a multi-channel pipette. The plate was immediately placed in a fluorescence microplate reader (SH-9000Lab, Hitachi, Tokyo, Japan), and the fluorescence intensities were recorded every 5 minutes for 60 minutes at 37°C with excitation/emission = 480 nm/520 nm. Seven calibration solutions using Trolox (0, 2.5, 5, 10, 25, 50, and 100 μM final concentrations) as the standard antioxidant were also carried out in the same run. All reaction mixtures were prepared in triplicate, and at least three independent runs were performed for each sample (Xiao and Miwa, 2017).

Scanning electron microscope analysis

Surface ultrastructure of CCA samples and gelatin from cold water fish skin were analyzed by scanning electron microscopy (SEM). Dry powder of CCAs and fish skin were mounted on stubs and then coated with thin layer of osmium in the osmium plasma coater (Neo osmium coater Neoc-AN, Meiwafosis Co., Ltd., Tokyo, Japan). The preparations were examined at 5.0 kV using a scanning electron microscope (S-4000, Hitachi).

Cell culture

PC12 (RCB 0009), a cell line derived from a transplantable rat pheochromocytoma was purchased from Riken Cell Bank, Ibaraki, Japan. PC12 cells were cultured in DMEM

Table 1 Components of simulated peptic juices for *in vitro* digestion

	Gastric juice	Duodenal juice	Bile
Inorganic compounds	1.57 mL NaCl 175.3 g/L	4.0 mL NaCl 175.3 g/L	3.0 mL NaCl 175.3 g/L
	0.3 mL NaH_2PO_4 88.8 g/L	4.0 mL $NaHCO_3$ 84.7 g/L	6.83 mL $NaHCO_3$ 84.7 g/L
	0.92 mL KCl 89.6g/L	1.0 mL KH_2PO_4 8g/L	0.42 mL KCl 89.6 g/L
	1.8 mL $CaCl_2{\cdot}2H_2O$ 22.2 g/L	0.63 mL KCl 89.6 g/L	20 μL HCl 37% g/g
	1.0 mL NH_4Cl 30.6 g/L	1.0 mL $MgCl_2$ 5 g/L	1.0 mL $CaCl_2{\cdot}2H_2O$ 22.2 g/L
	530 μL HCl 37% g/g	18 μL HCl 37% g/g	
		0.9 mL $CaCl_2{\cdot}2H_2O$ 22.2 g/L	
Organic compounds	0.34 mL urea 25 g/L	0.4 mL urea 25 g/L	1.0 mL urea 25 g/L
	1.0 mL glucose 65 g/L		
	1.0 mL glucuronic acid 2 g/L		
	1.0 mL D(+)-glucosamine ydrochloride 33 g/L		
Others	0.1 g BSA	0.1 g BSA	0.18 g BSA
	0.1 g pepsin	0.3 g trypsin	0.6 g bile salt
	0.3 g mucin	0.05 g amano lipase PS	
pH	1.07±0.07	7.8±0.2	8.0±0.2

BSA: Bovine serum albumin.

(high glucose) medium supplemented with 10% FBS at 37°C in a humidified tissue culture incubator with 5% CO_2 and 95% O_2 according to the previous report (Sakagami et al., 2017). For neuronal differentiation, PC12 cells were cultured in neuronal induction media [DMEM (high glucose) supplemented with NGF (50 ng/mL)] for 3–7 days.

Hydrogen peroxide treatment

Hydrogen peroxide (H_2O_2; 30%) (081-04215, FUJIFILIM Wako Pure Chemical Co., Japan) was diluted first in DDW then culture medium just prior to each experiment. PC12 cells (3×10^4/well) were seeded in 24-well plates and cultivated overnight. Then, PC12 cells were incubated with 200 μM H_2O_2 for 1 hour. After 1 hour of exposure, cells were rinsed and further cultivated in normal culture medium without H_2O_2 for another 4–5 days. Control cells were similarly treated without H_2O_2.

Cell viability assay

Cell viability in PC12 cells were measured by PrestoBlue® Assay according to the manufacturer's protocol. At the end of cultivation, PC12 cells were incubated for 3 hours at 37°C with fresh medium supplemented with 10% PrestoBlue® (v/v; Thermo Fisher Scientific, Tokyo, Japan; A13261). The PrestoBlue® reduction by the cells expressed as fluorescence intensity units was measured on a microplate reader (SH-9000Lab, Hitachi) with excitation/emission = 560 nm/590 nm.

Acetylcholinesterase activity assay (*Fluorometric assay)*

Acetylcholinesterase (AchE) activity of PC12 cells was measured by a fluorometric assay (Rieger et al., 1980). Briefly, PC12 cells were seeded in 24-well plates (30,000 cells/well) and then differentiated into neuronal-like cells by NGF (50 ng/mL). At the end of cultivation, after being washed twice by PBS (–) (PBS without Ca and Mg), PC12 cells were incubated with 5.6 mM acetylthiocholine iodide 40 μL in 360 μL of buffer I (0.12 M NaCl, 0.2% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH7.5) at room temperature for 2 hours. Protein concentrations of the cell lysate were measured with a protein quantification kit (Protein Quantification Kit-Rapid, Dojindo Molecular Technologies, Inc., Kumamoto, Japan). 20 μL of each cell lysate with the same concentration of protein was transferred into a 96-well plate and incubated with 160 μL of buffer II (1 mM EDTA, 0.2% Triton X-100, 50 mM acetate buffer; pH 5.0) and 20 μL 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (0.4 mM, in acetonitrile) at room temperature. After 1 hour of incubation, fluorescence intensity was measured at excitation/emission = 365 nm/450 nm using a microplate reader (SH-9000Lab, Hitachi).

Colorimetric assay (Ellman's assay)

We also performed colorimetric assay to determine cholinesterase activities according to the report by Ellman et al. (1961). Briefly, 10 μL enzyme (or enzyme-sample mixture) solution was mixed with 180 μL Ellman's reagent [0.5 mM 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) in 0.1 M phosphate buffer, pH 7.4] and incubated for 30 minutes at room temperature. Then 10 μL substrate [acetylthiocholine iodide (20 mM in double distilled water)] was added into the mixed solution and further incubated for another 10–30 minutes at room temperature. The absorbance was measured at 405 nm using a microplate reader (SH-9000Lab, Hitachi).

Whole-cell patch recording

PC12 cells were seeded onto glass coverslips and differentiated into neuronal cells by NGF-treatment with or without enzyme-digested *Colla Corii Asini* (CCAD) (0.5 mg/mL) for 6 days. At the end of the neural differentiation, whole-cell patch clump recordings were performed to single cells using an Axopatch 200B amplifier (Molecular Devices, Tokyo, Japan) at room temperature (21–23°C). Data were sampled at 20 kHz and digitized with a Digidata 1440A interface and recorded by pCLAMP 10.6 software (Molecular Devices). Data were low-pass filtered at 5 kHz. Patch pipettes were pulled from borosilicate glass capillaries on a NARISHIGE PC-10 puller and had resistances of 2–3 MΩ. Series resistance was compensated by at least 80%. For recoding action potentials, ramp protocol was applied by using a current clamp mode with 10 nA maximal amplitude. The bath solution was prepared with NaCl, 160 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; glucose, 10 mM; and HEPES, 10 mM in DDW; adjusted to pH 7.4 with NaOH. The pipette solution was prepared with K-gluconate, 130 mM; KCl, 30 mM; EGTA, 10 mM; $CaCl_2$, 1 mM; Mg-HTP, 2 mM; and HEPES, 10 mM; adjusted to pH 7.3 with KOH (Xiao et al., 2017).

$A\beta_{1-42}$ treatment

$A\beta_{1-42}$ (AS24224, AnaSpec, Inc., Fremont, CA, USA) was first solved in 1% NH4OH and then immediately diluted by PBS (–) at 100 μM and stored at –20°C until use according to the product data sheet. After 4 days of neuronal induction, PC12 cells were treated with $A\beta_{1-42}$ in neuronal inductive medium at 1 μM for 48 hours.

Enzyme-linked immunosorbent assay

To determine the levels of $A\beta_{1-42}$ in PC12 cells, samples were analyzed using a commercially available oligomeric amyloid-β ($_{1-42}$) enzyme-linked immunosorbent assay (ELISA) Kit, (BEK-2215-1P, Biosensis pty. Ltd., CA, USA). At the end of cultivation, PC12 cells were homogenized in 100 μL RIPA lysis buffer (WSE-7420 EzRIPA Lysis kit, ATTO Co., Tokyo, Japan) to obtain the extracts. Protein concentrations of the extracts were measured with a protein quantification kit (Protein Quantification Kit-Rapid, Dojindo Molecular Technologies, Inc.). For each ELISA, cell extracts (with the same concentration of protein) were diluted to bring the expected concentration within the range of the standard curve and reacted with the first and secondary antibodies, streptavidin-HRP and detection solution according to the manufacture's instruction. The reaction was stopped using the stop solution (from the ELISA kits) and absorbance was read at

450 nm using a microplate reader (SH-9000Lab, Hitachi) (Xiao et al., 2019b).

Neprilysin assay

Neprilysin activity of PC12 cells was measured using a commercially available SensoLyte Neprilysin Fluorimetric Activity Assay Kit (AS-72223, AnaSpec, Inc.). At the end of cultivation, PC12 cell extracts (with the same concentration of protein) were diluted to bring the expected concentration within the range of the standard curve and reacted with 5-FAM/QXL™-520 Neprilysin substrate according to the manufacture's instruction. After 60 minutes of incubation, fluorescence intensity was measured at excitation/emission = 490 nm/520 nm using a microplate reader (SH-9000Lab, Hitachi, Japan).

Statistical analysis

Statistical analysis was performed similarly to our previous report (Xiao et al., 2017). All data, expressed as the mean ± SD, were processed statistically by GNU PSPP Statistical Analysis Software (version 0.8.2-gad9374) (https://www.gnu.org/software/pspp/) and the OpenStat program by Bill Miller. For statistical analysis, one-way analysis of variance followed by the least significant difference test (equal variances assumed) or the Dunnett's T3 test (equal variances not assumed) was used. Statistical significance was considered when $P < 0.05$. All experiments were repeated 3–5 times independently.

Results

Effect of storage term on antioxidative activity of CCA

We used ORAC assay to test antioxidative activity of CCA and compared with gelatins from the skin of porcine, bovine and cold water fish. As shown in **Figure 1A**, at 1.0 mg/mL, gelatins from porcine, bovine and fish possessed equivalent value of the standard antioxidant Trolox at 44.7, 58.9 and 108.6 μM, respectively. Compared to them, CCA (manufactured in 2013) bore the highest equal value of Trolox at 207.6 μM. According to the ancient clinical observation, if CCA is stored under good conditions (dryness and no fungal growth), the longer it is stored, the better therapeutic effects it has. So we compared ORAC capacity of CCAs manufactured in different years. **Figure 1B** showed that at 0.5 mg/mL, ORAC capacity of CCAs exhibited a storage term-dependent manner. CCA manufactured in 2008 possessed the highest ability to scavenge peroxyl radicals (ROO·) derived from AAPH. Because in the human body, gelatins are digested into amino acids by the digestive system, we simulated the process of digestion of CCAs and fish gelatin *in vitro* using the mixed digestive enzymes (**Table 1**). We then measured ORAC capacity of the digested CCAs and fish gelatin. The result showed that ORAC capacity of digested CCAs also exhibited a storage term-dependent manner. Digested fish gelatin showed the lowest ORAC capacity compare to digested CCAs.

Effect of storage term on ultrastructural changes of CCAs and fish gelatin

We compared the surface ultrastructure of CCA manufactured in different years. **Figure 2** showed that the longer CCAs were stored, the smoother surface they had. Compared to CCAs, gelatin from cold water fish exhibited a rough surface. It has been reported that long-term storage could cause changes of amino acids in dried blood spots (Strnadová et al., 2007). It is possible that long-term storage causes concentrations of some amino acids in CCA to change, resulting in alteration of the surface ultrastructure of CCA.

Effect of digested CCA on H_2O_2-induced neuronal cell death

To further examine the effect of CCA on neuronal protection, we used NGF-differentiated PC12 neuron-like cells to perform the experiments. Because digested CCA manufactured in 2008 showed the highest ORAC capacity, we used CCAD 2008 to treat the cells for the rest of the study. As shown in **Figure 3A**, after 4 or 5 days of NGF (50 ng/mL) treatment, PC12 cells differentiating into neuronal cells (cells with at least one neurite with a length equal to the cell body diameter) as same as the previous report (Das et al., 2004). One-hour exposure of 200 μM H_2O_2 caused cell death resulting in cell viability decreased to 57% of that in cells without exposure. In contrast, cell viabilities of 0.5 mg/mL FGD- or CCAD-pretreated PC12 cells were significantly increased to 76% and 80%, respectively. The data suggests that both FGD and CCAD could protect neuronal-like PC12 cells from H_2O_2-induced injuries. CCAD showed significantly better protection than FGD ($P < 0.01$) (**Figure 3B**). Additionally both FGD- and CCAD-pretreated PC12 cells exhibited long neurites suggesting that FGD and CCAD did not obstruct neuronal differentiation (**Figure 3A**).

Effect of CCAD on AchE activity in PC12 neuronal cells

Numeral clinical evidence showed that AchE inhibitors, such as donepezil, can interfere with the progression of Alzheimer's disease (AD) (Sugimoto et al., 2000). We therefore added donepezil (10 μM), FGD (0.5 mg/mL) or CCAD (0.5 mg/mL) to PC12 cells during NGF differentiation to test their effects on AchE activity. The concentrations we chose did not decrease cell viability of NGF-differentiated PC12 cells (data not shown). As shown in **Figure 4A**, compared to non-differentiated cells (NC), NGF-differentiated PC12 cells exhibited significantly higher AchE activity. In donepezil-treated PC12 cells, the AchE activity was decreased to 14.3% of that in non-treated cells (NGF). Both FGD and CCAD treatments also significantly decreased AchE activity in PC12 cells to 89.1% and 74.5%. CCAD showed significantly better AchE inhibition than FGD ($P < 0.01$). However, our cell-free AchE assay showed that neither CCAD nor FGD has a direct inhibitory effect on AchE activity (**Additional Figure 1B**), suggesting that CCAD and FGD inhibit AchE activity in PC12 cells through affecting cellular functions.

As well known, AchE is an essential enzyme to maintain

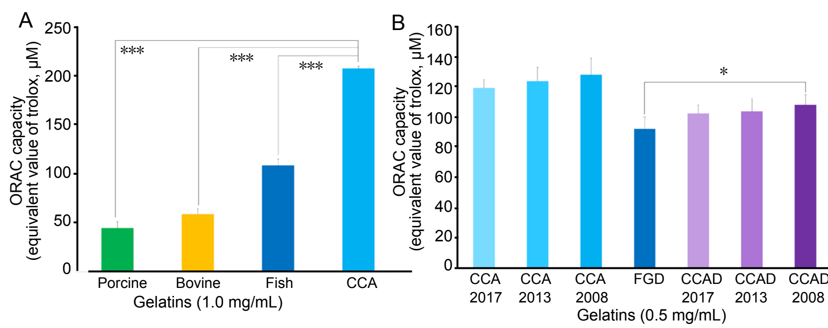

Figure 1 Peroxyl radical (ROO·) scavenging activity of *Colla Corii Asini* (CCA).
Peroxyl radical scavenging activity (ORAC capacity) of CCA was measured by ORAC assay as described in Materials and Methods. (A) ORAC capacity of porcine skin gelatin, bovine skin gelatin, gelatin from cold water fish skin and CCA manufactured in 2013 at 1.0 mg/mL. (B) ORAC capacity of CCA manufactured in 2017, 2013 and 2008 and digested fish gelatin (gelatin from cold water fish skin) and CCA manufactured in 2017, 2013 and 2008 at 0.5 mg/mL. Digested gelatins were all filtered through 0.22 μm filters before ORAC assay. CCAD: Enzyme-digested CCA; FGD: enzyme-digested fish gelatin; ORAC: oxygen radical absorbance capacity. Experiments were repeated 3–5 times independently. $*P < 0.05$, $***P < 0.001$ (one-way analysis of variance followed by the least significant difference test).

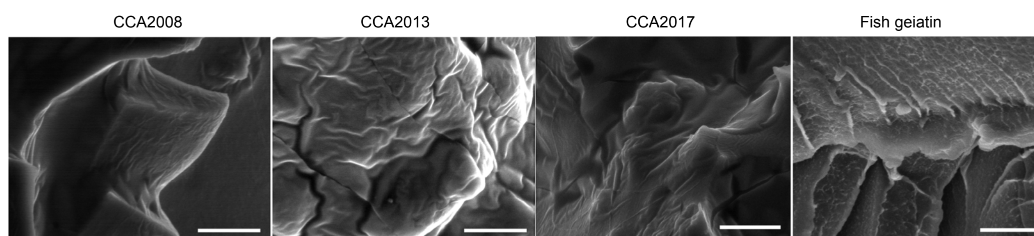

Figure 2 Ultrastructure of CCAs and fish gelatin.
Scanning electron microscopy (SEM) images of CCA (manufactured in 2008, 2013 and 2017) samples and fish gelatin powder. The samples were mounted on stubs and then coated with a thin layer of osmium. The surfaces of samples were observed (original magnification: 10,000×) under the scanning electron microscope. Scale bars: 1 μm. CCA: *Colla Corii Asini*.

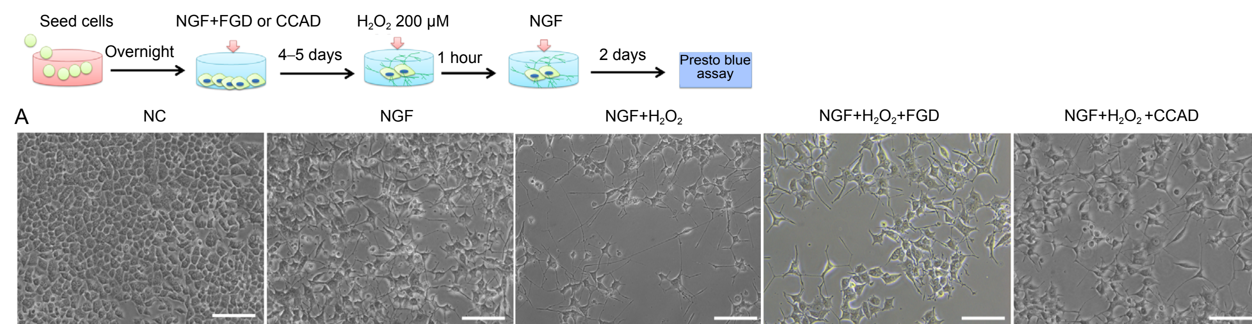

Figure 3 Effect of CCAD on H_2O_2-induced cell death in neuronal-like PC12 cells.
Top panel shows experimental setup. PC12 cells were seeded into 24-well plates at 30,000 cells/well in the maintenance medium and cultivated overnight. The next day cells were treated with H_2O_2 (200 μM) for 2 hours. After rinse out of H_2O_2, cells were treated with NGF (50 ng/mL) and FGD or CCAD at 0.5 mg/mL. Cells were further cultivated for 6 days. At the end of cultivation, cell viability was measured by presto blue assay. (A) Images of PC12 cells were observed by a phase-contrast microscope. Images were taken at the end of cultivation. Magnification: 200×. Scale bars: 25 μm. (B) Results from presto blue assay. Experiments were repeated 3–5 times independently. $***P < 0.001$, *vs.* NGF + H_2O_2; $††P < 0.01$, *vs.* NGF + H_2O_2 + FGD (one-way analysis of variance followed by the least significant difference test). CCAD: Enzyme-digested *Colla Corii Asini*; FGD: enzyme-digested fish gelatin; NC: negative control; NGF: nerve growth factor.

the homeostasis of cholinergic nervous system. Dysfunction of AchE can induce abnormal accumulation of the neurotransmitter acetylcholine that results in severe problems. There is evidence showing that the neuronal function and activity of AchE are impaired in AD patients. The benefits of AchE inhibitors are limited (Campanari et al., 2017). It has been reported that H_2O_2 could damage the enzyme structure of AchE by oxidation and reduce its activity (Schallreuter et al., 2004; Molochkina et al., 2005). Our data showed that H_2O_2 could directly inhibit the activity of AchE in a dose-dependent manner (**Additional Figure 1A**). Both CCAD and FGD could significantly restore H_2O_2-inhibited AchE activity whereas donepezil could not (**Additional Figure 1B**). We further checked the effect of CCAD on AchE activity in H_2O_2-treated PC12 cells. **Figure 4B** showed that 1-hour exposure of H_2O_2 (200 μM) resulted in a dramatic decrease of AchE activity in both undifferentiated cells (NC) and NGF-differentiated PC12 cells, suggesting that oxidative

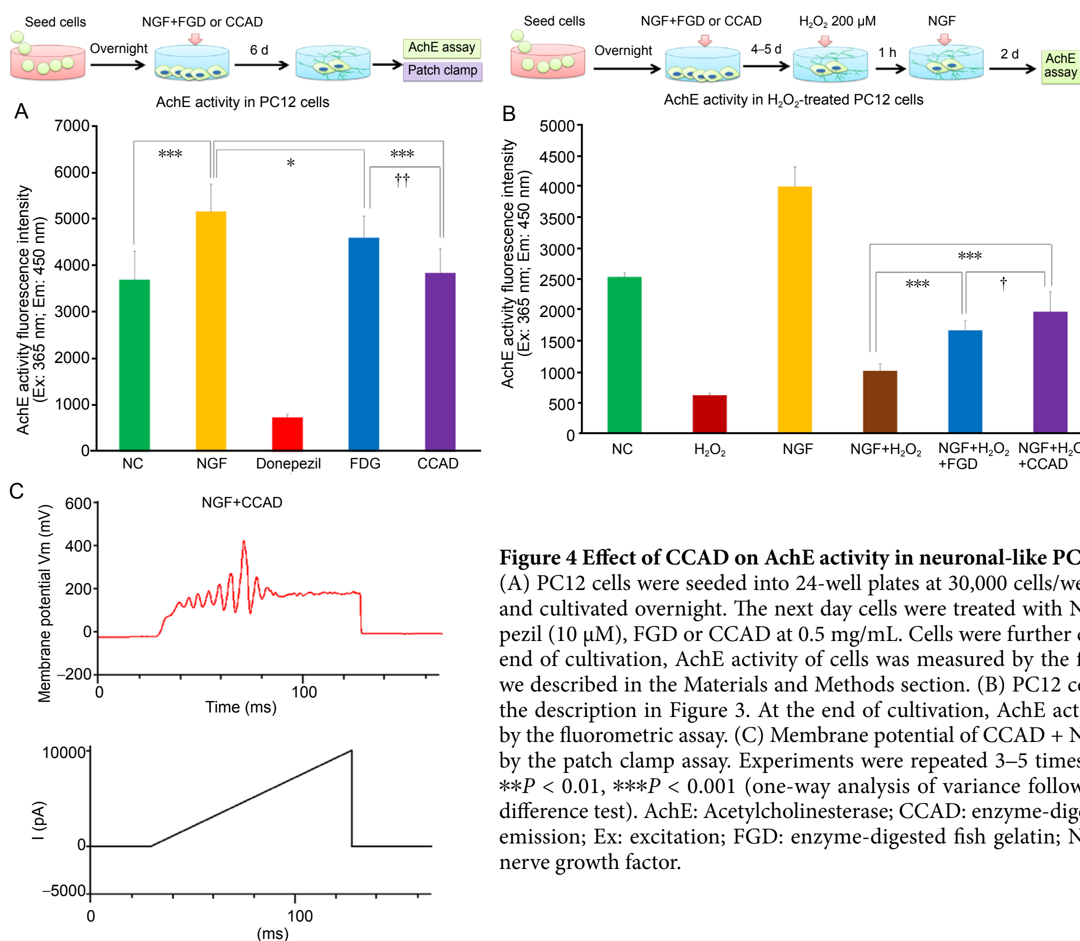

Figure 4 Effect of CCAD on AchE activity in neuronal-like PC12 cells.
(A) PC12 cells were seeded into 24-well plates at 30,000 cells/well in maintenance medium and cultivated overnight. The next day cells were treated with NGF (50 ng/mL) and donepezil (10 μM), FGD or CCAD at 0.5 mg/mL. Cells were further cultivated for 6 days. At the end of cultivation, AchE activity of cells was measured by the fluorometric AchE assay as we described in the Materials and Methods section. (B) PC12 cells were treated as same as the description in Figure 3. At the end of cultivation, AchE activity of cells was measured by the fluorometric assay. (C) Membrane potential of CCAD + NGF-treated cells was tested by the patch clamp assay. Experiments were repeated 3–5 times independently. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (one-way analysis of variance followed by the least significant difference test). AchE: Acetylcholinesterase; CCAD: enzyme-digested *Colla Corii Asini*; Em: emission; Ex: excitation; FGD: enzyme-digested fish gelatin; NC: negative control; NGF: nerve growth factor.

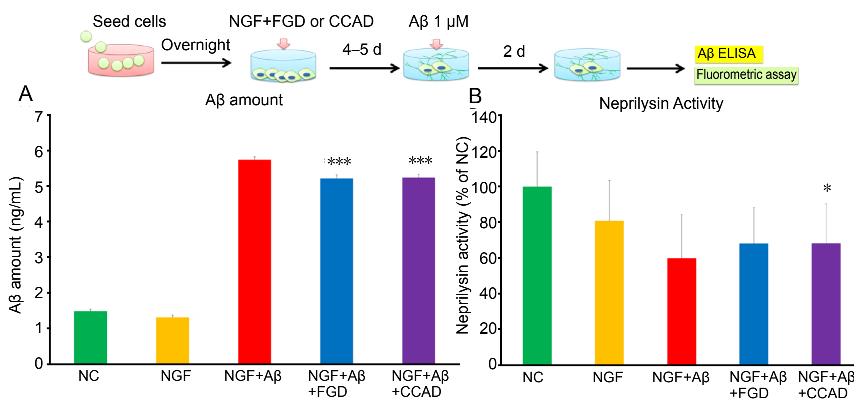

Figure 5 Effect of CCAD on Aβ clearance in neuronal-like PC12 cells.
PC12 cells were seeded into 24-well plates at 30,000 cells/well in maintenance medium and cultivated overnight. The next day, cells were treated with NGF (50 ng/mL) and FGD or CCAD at 0.5 mg/mL and cultivated for 4 days. Then cells were treated with Aβ at 1 μM for 2 days. At the end of cultivation, ELISA (A) and neprilysin activity fluorometric assay (B) were performed. Experiments were repeated 3–5 times independently. $*P < 0.05$, $***P < 0.001$, *vs.* NGF + Aβ (one-way analysis of variance followed by the least significant difference test). Aβ: Amyloid beta; CCAD: enzyme-digested *Colla Corii Asini*; ELISA: enzyme-linked immunosorbent assay; FGD: enzyme-digested fish gelatin; NC: negative control (without NGF-treatment); NGF: nerve growth factor.

stress severely injured normal cellular functions. However, pretreatments with 0.5 mg/mL FGD or CCAD significantly restored AchE activity, especially CCAD. Our data suggested that CCAD could protect PC12 cells from oxidative stress-induced impairment of cellular functions. Therefore, CCAD has bidirectional effects on AchE activity in neuronal cells.

We further tested neuronal function of NGF-differentiated PC12 cells supplied with CCAD by using patch clamp technique. As shown in **Figure 4C**, PC12 cells treated with both NGF and CCAD exhibited the capacity to produce action potentials, suggesting that these cells were functional neurons.

Effect of CCAD on Aβ clearance in PC12 neurons

Aβ accumulation is a hallmark for AD. Accelerating Aβ clearance may slow the rate of AD onset. Neprilysin is the dominant Aβ peptide-degrading enzyme in the brain (El-Amouri et al., 2008). Hence, we added human $Aβ_{1–42}$ peptide to NGF-differentiated PC12 cells and checked the effects of CCAD on the amount of Aβ and neprilysin activity. As shown in **Figure 5**, without Aβ treatment (1 μM, 48 hours), there were only 1.49 and 1.32 ng/mL Aβ in the non-differentiated cells (NC) and NGF-differentiated cells, respectively. With Aβ treatment, Aβ amount was increased to 5.74 ng/mL. Pretreatments with FGD or CCAD significantly decreased Aβ amount to 5.22 ng/mL and 5.24 ng/mL,

respectively ($P < 0.001$). Aβ treatment decreased neprilysin activity in NGF-differentiated PC12 cells whereas FGD or CCAD pretreatment increased neprilysin activity. CCAD especially showed significant effects ($P < 0.05$; **Figure 5B**). These data suggest that CCAD and FGD could accelerate Aβ clearance at the cellular level.

Discussion

In the present study, our data demonstrated that CCA has abilities to prevent H_2O_2-induced neuronal-like PC12 cell death and functional damage, reduce AchE activity, and accelerate Aβ clearance. Since the main compounds of CCA are collagen-derived amino acids (Wong et al. 2014), the neuroprotection of CCA probably is due to amino acids. According to ancient Chinese medical books (such as the book for herbal medicine, Bencao cong xin, published in 1757), the therapeutic effects of CCA are increased and the side effects are decreased in a storage term-dependent manner. Our ORAC assay results presented that the antioxidative activity of CCAs increased in a storage term-dependent manner. CCA that was in storage for 11 years (manufactured in 2008) exhibited the highest antioxidative activity. SEM analysis showed that older CCA has smoother surface ultrastructure. According to the previous report, the levels of amino acids decrease during the long-term storage. However, the degenerative rates of each amino acid are different (Strnadová et al., 2007). As a result, the balance of the overall amino acids could be changed. We think that the increased antioxydative activity and the ultrastructure change were probably due to the changed balance of amino acids in CCA during storage. The dynamic changes of amino acids and other compounds were examined.

Oxidative stress-caused damage to the brain has been recognized as a contributing factor in the progression of AD. Oxidative stress is associated with over-produced reactive oxygen species (ROS) including superoxide anion radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO·), nitric oxide (NO), and peroxynitrite ($ONOO^-$) (Tönnies et al., 2017). As one of the most important ROS, H_2O_2 mediates Aβ toxicity to neuronal cells (Behl et al., 1994). Our data showed that just treating neuronal-like PC12 cells with H_2O_2 (200 μM) for only 1 hour can cause severe damage to the cells. The cells suffered from growth suppression and function loss (they were not able to produce the physiological level AchE). Both FGD and CCAD protected PC12 cells from H_2O_2-induced oxidative damages, especially CCAD.

Because the declined mental function associated with AD is related to the central cholinergic deficit, so acetylcholinesterase inhibitors (AchEIs) have been used to improve the symptoms of AD patients. Among AchEIs, donepezil exhibits the best pharmacological profile in terms of cognition, behavior, and activities of the daily living in both moderate and severe AD (Cacabelos, 2007). Our data confirmed that donepezil at a non-cytotoxic concentration (10 μM) could markedly decrease AchE activity in neuronal-like PC12 cells. Both FGD and CCAD also showed inhibitory effects on AchE activity although they are not as efficient as donepezil.

Extracellular and intracellular oligomers of Aβ were demonstrated to be highly synaptotoxic, especially $A\beta_{1-42}$ and $A\beta_{1-40}$ (Zago et al., 2012). Aβ accumulation in the brain associated with neuroinflammation, synaptic loss, impaired neuronal function, and ultimately debilitating cognitive decline (Selkoe, 2002; Querfurth and LaFerla, 2010). Therefore, reducing Aβ levels in the brain, either by inhibiting its production/aggregation or by increasing its clearance, are considered as an advantageous strategy in preventing the development of AD (Zuroff et al., 2017). In the Aβ protein clearance and degeneration pathway, neprilysin plays an important role (Baranello et al., 2015). Neprilysin degrades Aβ peptide not only in the monomeric form but also the pathological oligomeric forms (Aβ peptides 1–40 and 1–42) (Shirotani et al., 2001; Kanemitsu et al., 2003). Neprilysin level in AD patients is declined (Hellström-Lindahl et al., 2008). Age-related oxidative damage to neprilysin in the brains of AD patients was more severe than that in age-matched normal controls (Wang et al., 2003). In this study, we treated neuronal-like PC12 cells with $A\beta_{1-42}$ oligomer. The results showed that $A\beta_{1-42}$ oligomer decreased the level of its degrading enzyme, neprilysin, suggesting $A\beta_{1-42}$ itself could suppress the ability of PC12 to eliminate the oligomer. CCAD and FGD showed significant effects on Aβ clearance: they both decreased Aβ accumulation and increased neprilysin level in neuronal-like PC12 cells, especially CCAD.

The limitation of this study was that PC12 cells can not completely or really represent the pathogenic lesions of AD and the underlining mechanism of the neuroprotective effects of CCA has not yet been explored. Also we need to know if the digested CCA can permeate and cross the blood-brain barrier. However, we first demonstrated that CCA could 1) offer neuroprotection against oxidative stress-induced neuronal-like cell death; 2) accelerate Aβ clearance at the cellular level; 3) inhibit AchE activity to assumedly enable an increase in neurotransmitter acetylcholine. These properties of CCA might be able to affect the brain tissue in multiple ways to delay the onset of AD.

In conclusion, this study shows that CCAD can alleviate AD-associated pathogenic lesions *in vitro* through multiple ways. CCA can be explored as a therapeutic reagent for preventing the progression of AD.

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Additional file:
Additional Figure 1: *Direct effect of CCAD on AchE activity.*

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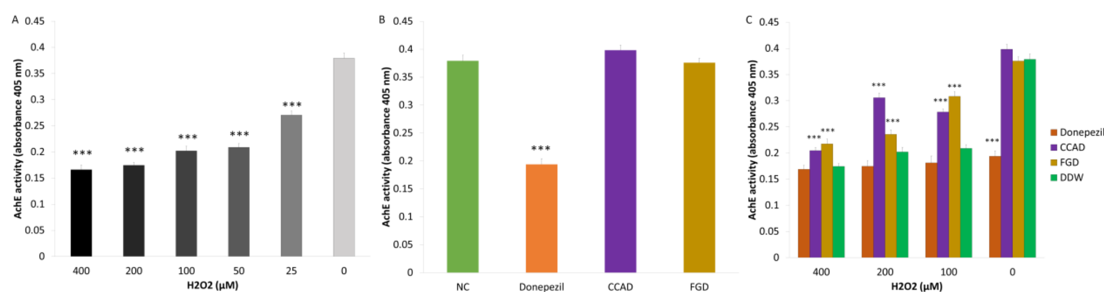

Additional Figure 1 Direct effect of CCAD on AchE activity.

AchE activity was measured by the colorimetric assay. (A) 5 μL AchE (1 unit/mL) and 5 μL H2O2 at different concentrations (400, 200, 100, 50, 25, and 0 μM) were mixed and incubated for 10 minutes before the reaction. The solutions were then reacted with Ellman's reagent and the substrate. (B) 5 μL AchE (1 unit/mL) and 5 μL samples (NC, DDW; donepezil, 10 μM; CCAD, 0.5 mg/mL; FGD, 0.5 mg/mL) were mixed and incubated for 10 minutes before the reaction. (C) 5 μL AchE (1 unit/mL, and the final concentration is 0.5 unit/mL), 2.5 μL samples (donepezil, 10 μM; CCAD, 0.5 mg/mL; FGD, 0.5 mg/mL), 2.5 μL H2O2 at 400, 200 and 100 μM were mixed and incubated for 10 minutes before the reaction. As the positive control, 2.5 μL DDW was mixed with 2.5 μL H_2O_2 at differentiations. AchE: Acetylcholinesterase; CCAD: enzyme-digested *Colla Corii Asini;* DDW: double distilled water; FGD: enzyme- digested fish gelatin; NC: negative control.