

Investigation of the neuroprotective effects of crocin via antioxidant activities in HT22 cells and in mice with Alzheimer's disease

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Abstract. Due to its complex pathogenesis, the prevention and therapization of Alzheimer's disease (AD) remains a serious challenge. Crocin, the main compound isolated from *Crocus sativus* L., demonstrates various pharmacological activities including anti-apoptotic properties. The present study investigated the neuroprotective effect of crocin and the underlying mechanisms. In L-glutamate-damaged HT22 cells, 3-h crocin pretreatment strongly enhanced the HT22 cell viability, reduced the apoptotic rate, mitigated mitochondrial dysfunction, suppressed intracellular reactive oxygen species (ROS) accumulation and Ca²⁺ overload compared with untreated cells. Additionally, crocin significantly decreased the expression levels of Bax, Bad and cleaved caspase-3 and

increased the expression levels of B-cell lymphoma-extra large, phosphorylated (P-) protein kinase B and P-mammalian target of rapamycin compared with untreated cells. In mice with AD induced by D-galactose and aluminum trichloride, crocin substantially improved the cognition and memory abilities of the mice as measured by their coordination of movement in an open field test, and reduced their escape time in the Morris water maze test compared with untreated mice. Biochemical analysis confirmed that crocin was able to reduce the Aβ₁₋₄₂ content in the mouse brains, increase the levels of glutathione peroxidase, superoxide dismutase, acetylcholine and choline acetyltransferase, and reduce the levels of ROS and acetylcholinesterase in the serum, cerebral cortex and hypothalamus compared with untreated mice. Immunohistochemical analysis demonstrated that crocin reduced Aβ₁₋₄₂ deposition in the hippocampus of the brains of treated mice compared with untreated mice. In conclusion, crocin demonstrates good prospects in the treatment of AD through the oxidative stress-associated apoptosis signaling pathway.

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Abbreviations: Ach, acetylcholine; AchE, acetylcholinesterase; Akt, protein kinase B; AlCl₃, aluminum trichloride; Aβ, amyloid β; Bcl-xL, B-cell lymphoma-extra large; ChAT, choline acetyltransferase; D-gal, D-galactose; DMEM, Dulbecco's modified Eagle's medium; Fluo-4, AM, 1-[2-Amino-5-(2,7-difluoro-6-hydroxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy) ethane-N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester; GSH-Px, glutathione peroxidase; HRP, horseradish peroxidase; HT22, Hippocampal neuronal cell line; L-Glu, L-glutamate; MMP, mitochondrial transmembrane potential; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; ROS, reactive oxygen species; SOD, superoxide dismutase; TBS, t-butyldimethylsilyl; TBST, TBS + Tween

Key words: Alzheimer's disease, crocin, oxidative stress, apoptosis, cholinergic neurotransmitter

Introduction

Alzheimer's disease (AD), a progressive degenerative disease of the nervous system, is characterized by a substantial decline in cognitive function and poor prognosis. Intracellular neurofibrillary tangles and extracellular senile plaques, particularly aggregated amyloid-β titanium and hyper-phosphorylated tau, are considered typical markers of AD histopathology (1). Although the exact molecular mechanism of neurodegeneration in AD remains unclear, through basic and clinical studies (2,3), oxidative stress and neuronal apoptosis have been reported to serve a pivotal function in the process of AD. Oxidative stress, resulting in an imbalance of anti-oxidation and pro-oxidation, may promote amyloid β (Aβ) aggregation accompanied by the over-accumulation of inflammatory reactive oxygen species (ROS) (4). Additionally, hyper-levels of ROS tend to enhance the dissipation of the mitochondrial transmembrane potential (MMP). In a short feedback loop, the increased permeability of mitochondria further enhances the over-releasing of ROS and other factors, including cytochrome c, into the cytoplasm, which triggers the cell apoptosis program (5,6). Abnormally high levels of neurons damaged

by glutamate, a central-nervous neurotransmitter, are closely associated with ROS accumulation (7).

The global impact of AD is rapidly expanding (8), as >40 million patients globally have been reported, which is expected to increase to 70 million by 2030 (8). Therefore, the prevention and therapeutization of AD is an urgent challenge. Unfortunately, clinical trials of AD drugs have a high failure rate, and the majority of them exert various adverse effects including loss of appetite, gastrointestinal discomfort, difficulty sleeping and muscle spasms (9). Herbal compounds have been considered as potential agents for the prevention of AD (10,11). Crocin, the main component of *Crocus sativus* L. extract, is a yellow carotenoid that has been reported to exert anti-inflammatory (12), anti-depressant (13), memory improvement (14) and anti-apoptotic properties (15). The structure of crocin is presented in Fig. 1. In PC12 cells, crocin prevents cell apoptosis via increasing glutathione synthesis (16); meanwhile, in aging Wistar rats, crocin enhances their memory abilities, mainly through its anti-oxidant activities (17). In rats administered tramadol hydrochloride, crocin improves their learning and reduces memory impairment (18). However, no systematic study of the effects of crocin on AD, and its underlying mechanisms, has been reported.

In the present study, the neuroprotective effects of crocin against AD were investigated in L-glutamate (L-Glu)-induced HT22 apoptotic cells, and in aluminum trichloride (AlCl₃) and D-galactose (D-gal)-induced AD mice. The present study aimed to provide experimental evidence that crocin may be a candidate agent for the adjuvant treatment of AD.

Materials and methods

Cell culture. HT22 cells (cat. no. 337709; BeNa Culture Collection, Beijing, China), a mouse hippocampal neuronal cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% 100 µg/ml streptomycin and 100 units/ml penicillin (all Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a 5% CO₂ incubator to provide a humidified atmosphere (Thermo Fisher Scientific, Inc.).

MTT assay. HT22 cells cultured in 96-well plates, were pretreated with crocin (cat. no. B21336; purity ≥98%; Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) at doses of 0.5, 1, 2 and 4 µM for 3 h at 37°C and then incubated with 25 mM L-Glu for another 24 h at 37°C. The control cells were not treated with 25 mM L-Glu and crocin, and incubated for 24 h. A total of 5 mg/ml MTT (Merck KGaA, Darmstadt, Germany) was added for 4 h at 37°C, and then 100 µl dimethyl sulfoxide (Merck KGaA, Darmstadt, Germany) was added. Absorbance was analyzed using a Synergy™⁴ Microplate Reader at 490 nm (BioTek Instruments, Inc., Winooski, VT, USA).

Cell apoptosis assay. HT22 cells cultured in 6-well plates were pretreated with crocin at doses of 0.5 and 2 µM for 3 h at 37°C and then incubated with 25 mM L-Glu for 24 h at 37°C. Collect cells and resuspend in 100 µl 1% FBS, then incubated with Annexin V & Dead Cell Reagent (cat. no. 4700-1485, 100 tests/bottle) for 20 min at 25°C in the dark, and detected

using the Muse™ Cell Analyzer (EMD Millipore, Billerica, MA, USA).

MMP, intracellular ROS and Ca²⁺ measurement. HT22 cells were pretreated with crocin at doses of 0.5 and 2 µM for 3 h and then incubated with 25 mM L-Glu for 12 h. Cells were further incubated with 2 µM 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide staining (EMD Millipore, Billerica, MA, USA), 10 µM 2'-7'-dichlorodihydrofluorescein diacetate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or 2 µM Fluo-4-AM (Molecular Probes; Thermo Fisher Scientific, Inc.) for 20 min at 25°C in the dark to investigate the changes in MMP and the levels of intracellular of ROS and Ca²⁺, respectively. All fluoro-chrome were dissolve in serum-free DMEM. Subsequent to washing, the fluorescence intensities were detected using fluorescence microscopy (CCD camera, TE2000; Nikon Corporation, Tokyo, Japan).

Western blot analysis. HT22 cells were pretreated with crocin at doses of 0.5 and 2 µM for 3 h and then incubated with 25 mM L-GLU for 24 h. Cells were lysed for 0.5 h at 0°C with radio immunoprecipitation assay buffer containing 2% phenylmethanesulfonyl fluoride (Sigma-Aldrich; Merck KGaA) and 1% protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Subsequent to the detection of the protein concentration using the BCA kit (Merck KGaA) 40 µg lysates were separated using 12% dodecyl sulfate, sodium salt polyvinylidene fluoride membranes (0.45 µm; Merck KGaA). The membranes were blocked with 5% bovine serum albumin for 2 h at 4°C, and then incubated with primary antibodies as follows: B-cell lymphoma-extra large (Bcl-xL; 26 kDa; cat. no. ab32370), Bcl2 associated X, apoptosis regulator (Bax; 21 kDa; cat. no. ab32503), Bcl2 associated agonist of cell death (Bad; 23 kDa; cat. no. ab32445), cleaved caspase-3 (32 kDa; cat. no. ab2302), phosphorylated (P-) protein kinase B (Akt; phospho S473; 60 kDa S473; cat. no. ab18206), total (T)-Akt (60 kDa; cat. no. ab106693), P-mammalian target of rapamycin (mTOR; phospho S2448; 289 kDa S2448; cat. no. ab109268), T-mTOR (289 kDa; cat. no. ab83495) and GAPDH (36 kDa; cat. no. ab181602), which were purchased from Abcam (Cambridge, MA, USA) at 4°C overnight at a dilution of 1:2,000. Subsequent to three washes with TBST (consists of 500 ml TBS and 0.5 ml Tween-20), TBS (20X) is composed of 48.4 g Tris, 584 g NaCl and 900 ml distilled water (Beijing Chemical Works Beijing, China), the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G antibody (cat. no. SH-0032; Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) at 4°C for 4 h at a dilution of 1:2,000. An enhanced chemiluminescence kit (Merck KGaA) was used to visualize the protein bands under an imaging system (BioSpectrum600). Quantitation of the results was accomplished by ImageJ (x64) 1.48u software (National Institutes of Health, Bethesda, MD, USA).

AD mice model establishment and administration. The experiment was ethically approved by the Institution Animal Ethics Committee of Jilin University (Changchun, China; license no. 20160409). A total of 100 BALB/c mice (6-8 weeks; 18-20 g)

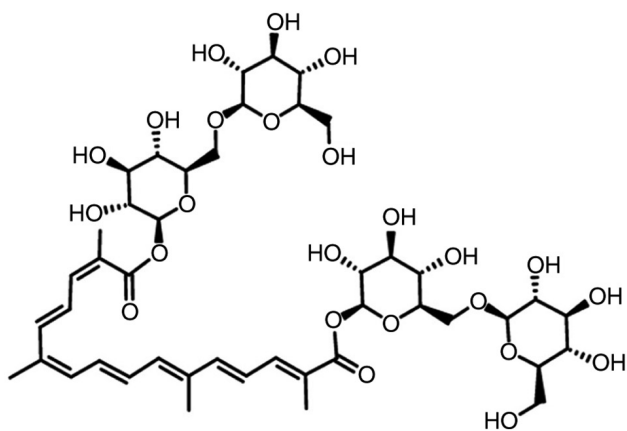


Figure 1. Structural formula of crocin.

were maintained at a standard 12:12 h light/dark cycle at $23 \pm 1^\circ\text{C}$. The mice were fed autoclaved standard chow *ad libitum*.

As described in a previous study (19), a total of 60 mice were randomly intraperitoneally injected with 120 mg/kg D-gal (Sigma-Aldrich; Merck KGaA) and intragastrically administrated with 20 mg/kg AlCl_3 (Sigma-Aldrich; Merck KGaA) once a day for 8 weeks, as the process of the establishment of the AD mice model. From the 5th week onwards, crocin-treated mice were intragastrically treated with 5 or 20 mg/kg crocin everyday ($n=20/\text{group}$); meanwhile, model mice were intragastrically administrated with saline ($n=20$). A total of 20 mice injected intraperitoneal and intragastrical with saline for 8 weeks served as the control group. Another 20 mice were intraperitoneally and intragastrically injected with saline throughout the whole experiment and intragastric treatment with 20 mg/kg crocin from the 5th week, which served as the crocin single treated mice group. If any mouse exhibited endpoint signs, including rapidly reduced bodyweight (loss of $>20\%$ bodyweight), they were euthanized using an intraperitoneal injection of 200 mg/kg pentobarbital. During the whole experiment period, the maximum body weight loss was $<8.3\%$ amongst all experimental mice.

Animal behavioral detection

Morris water maze test. As described in a previous study (19), a circular pool filled with 10 cm (depth) water ($22\text{--}24^\circ\text{C}$) containing 1 l milk was used to perform the water maze test. A total of 5 days prior to the form test, mice were trained for 5 min per day. The latency from the immersion of each mouse into the pool until they escaped onto the hidden platform was recorded. On the test day, mice were subjected to the pool in the same quadrant to identify the platform, and the time spent to locate the platform within 120 sec was recorded.

Open field test. A soundproof dark box was set with a bottom area of 30×30 cm, with a central region of 15×15 cm as the central area, and the remaining area as the surrounding area. On the test day, mice were placed in the fixed central position in the box, and their moving track and the time of exploring the central area within 5 min were observed and recorded.

Sample collection and biochemical detection. Blood of all experimental mice were collected from a caudal vein subsequent to the behavioral tests. All mice were euthanized by an intraperitoneal injection of 200 mg/kg pentobarbital. Following this, the brains and hypothalamus were immediately collected. The cerebral cortex, separated from the brains ($n=20/\text{group}$) and the hypothalamus ($n=20/\text{group}$) were homogenized in ice-cold phosphate buffered saline (PBS).

Subsequent to detecting the protein concentration using a BCA assay kit (Merck KGaA), the levels of acetylcholine (Ach; cat. no. CK-E20536), acetylcholinesterase (AChE; cat. no. CK-E93899), choline acetyltransferase (ChAT; cat. no. CK-E94456), super oxidase dismutase (SOD; cat. no. CK-E20348), glutathione peroxidase (GSH-Px; cat. no. CK-E92669) and ROS (cat. no. CK-E91516) in serum, the cerebral cortex and the hypothalamus, and $\text{A}\beta_{1-42}$ (cat. no. CK-E94157) in the serum and cerebral cortex were detected using enzyme-linked immunosorbent assay kits according to the manufacturers protocols (Shanghai Yuanye Biotechnology Co., Ltd.).

Histological examination. The fixed whole brain hemisphere, kidney and spleen were washed, gradient dehydration using 30, 50, 70, 80, 95 and 100% ethanol in sequence and finally embedded in paraffin at 25°C for 12 h, which were further sliced into $4\text{ }\mu\text{m}$ sections. Then, a hydrating gradient using 100, 95, 80, 70 and 50% ethanol, and distilled water were applied in sequence and sections were stained using hematoxylin-eosin (H&E) as previously described (20).

Immunohistochemistry. Brain tissues ($n=10/\text{group}$) were fixed with 4% formalin solution at 25°C for 24 h, gradient dehydration using 30, 50, 70, 80, 95 and 100% ethanol in sequence, washed in xylene and embedded in paraffin, and then sliced into $5\text{ }\mu\text{m}$ -thick sections. Slides were dewaxed, hydrated and boiled for 10 min in 10 mM sodium citrate buffer (pH 6) and cooled at 25°C for 30 min. Subsequent to incubation for 10 min in 3% hydrogen peroxide, sections were blocked with normal 10% goat serum for 30 min at 25°C and then incubated overnight at 4°C with primary antibodies against $\text{A}\beta$ (1:200; cat. no. ab32136; Abcam, Cambridge, UK). Subsequent to washing with PBS, the sections were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (cat. no. sc-3836; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at 25°C and then incubated with streptavidin-organism HRP complex (Shanghai BestBio Science, China) for 60 min at 25°C . The peroxidase conjugate was counterstained using 5% diaminobenzidine tetrahydrochloride solution as the chromogen and hematoxylin at 25°C for 5 min. Immunoperoxidase staining of $\text{A}\beta$ was observed using light microscopy (magnification $\times 100$; Olympus Corporation, Tokyo, Japan).

Statistical analysis. Data were expressed as the mean \pm the standard error of the mean. One-way analysis of variance followed by post-hoc multiple comparisons (Dunn's test) was used to determine statistical significance using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

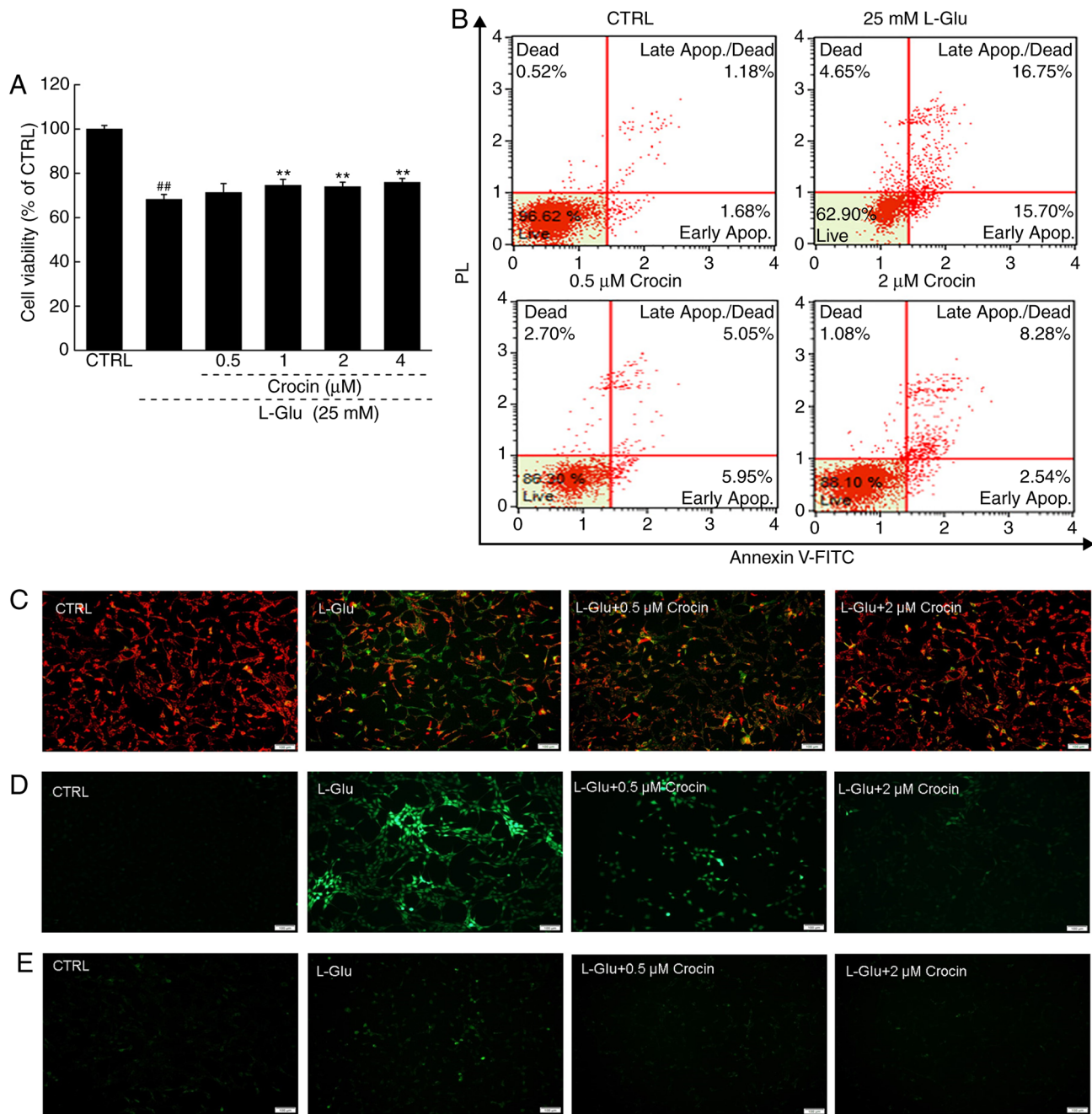


Figure 2. Crocin protected HT22 cells against L-Glu induced mitochondrial apoptosis. (A) Crocin enhanced cell viability in HT22 cells exposed to L-Glu for 24 h (n=10). ^{##}P<0.01 vs. CTRL. ^{**}P<0.01 vs. L-Glu-treated cells. (B) Crocin reduced the apoptosis rate in HT22 cells exposed to L-Glu for 24 h identified by analysis by Annexin V-FITC/PI staining (n=10). (C) Crocin restored L-Glu-induced MMP dissipation following 12 h co-incubation (n=10; scale bar, 100 μ m). (D) Crocin decreased the over-accumulation of ROS induced by 12 h L-Glu exposure (n=10; scale bar, 100 μ m). (E) Crocin prevented intracellular Ca^{2+} overload induced by 12 h L-Glu exposure (n=10; scale bar, 100 μ m). L-Glu, L-Glutamate; CTRL, control; FITC, fluorescein isothiocyanate; PI, propidium iodide; ROS, reactive oxygen species.

Results

Crocin protected HT22 cells against L-Glu-induced mitochondrial apoptosis. Crocin significantly increased cell viability in the cells exposed to L-Glu for 24 h compared with cells exposed to L-Glu alone (P<0.01; Fig. 2A). Among the HT22 cells exposed to L-Glu for 24 h, 32.5% were revealed to be apoptotic; in contrast, 3 h pre-incubation with crocin reduced the apoptosis rate to 10.8% (Fig. 2B).

An imbalance in MMP characterizes the early stage of mitochondrial injury (21). Compared with the L-Glu-damaged HT22 cells subsequent to 12 h of exposure, 3 h of pre-incubation

with crocin strongly restored the MMP dissipation, as indicated by the increased red fluorescence intensity and reduced green fluorescence intensity (Fig. 2C).

Crocin also strongly suppressed the hyper-levels of intracellular ROS compared with the L-Glu-damaged HT22 cells, as indicated by the reduced green fluorescence intensity (Fig. 2D); and intracellular ROS serves as an important mediator of cell damage (22).

The overload of Ca^{2+} caused by L-Glu is another factor reportedly associated with mitochondrial function (23). In the 12 h L-Glu-exposed HT22 cells, crocin strongly inhibited the overload of Ca^{2+} compared with the L-Glu-damaged HT22

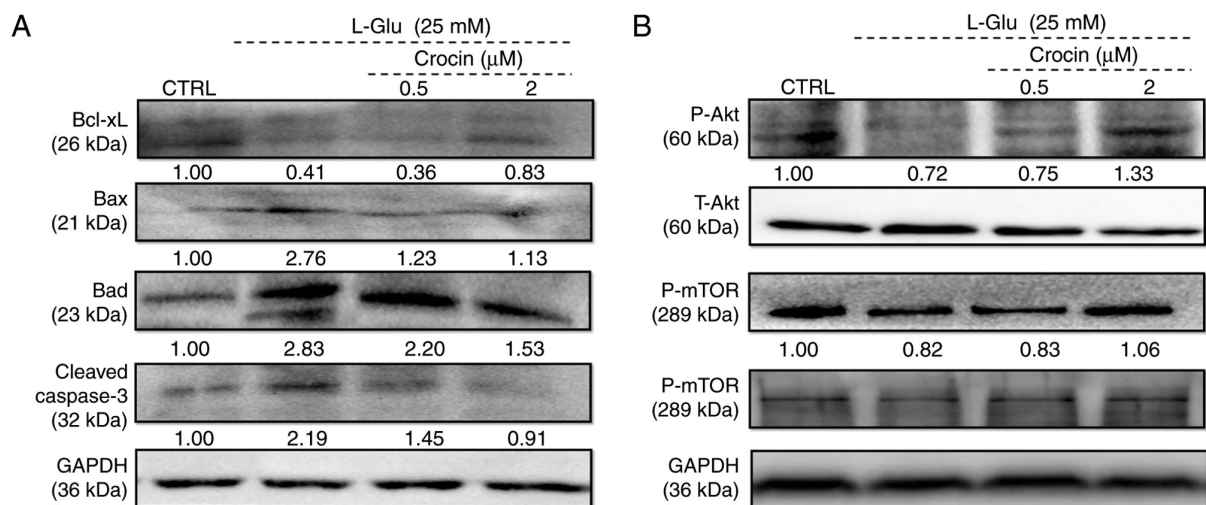


Figure 3. Crocin regulated the expression of anti- and pro-apoptotic proteins in HT22 cells exposed to L-Glu for 24 h. Expression of (A) Bcl-xL, Bax, Bad, cleaved caspase-3, (B) P-Akt, T-Akt, P-mTOR and T-mTOR were examined. Crocin increased the expression of Bcl-xL, P-Akt and P-mTOR, and reduced the expression of Bax, Bad and cleaved caspase-3. Quantification data were normalized using GAPDH and corresponding total proteins, and the mean fold of band intensity compared with the CTRL group was noted (n=6). L-Glu, L-glutamate; CTRL, control; Bcl-xL, B-cell lymphoma-extra large; Bax, Bcl2 associated X, apoptosis regulator; Bad, Bcl2 associated agonist of cell death; P-, phosphorylated; T-, total; Akt, protein kinase B; mTOR, mechanistic target of rapamycin.

cells, as indicated by the reduced green fluorescence intensity (Fig. 2E).

The expression of Bcl-2 family members directly influence mitochondrial function (24). In L-Glu-damaged HT22 cells, the low expression of Bcl-xL and high expression of Bax, Bad and cleaved caspase-3 compared with control cells were identified (Fig. 3A). Comparatively, 3 h of pre-incubation with crocin substantially reversed these changes to anti- and pro-apoptotic protein levels, and even restored them to their standard levels (Fig. 3A).

Akt/mTOR signaling is involved in the pro-survival and anti-apoptosis of neuronal protection (25). Following 24 h of exposure in HT22 cells, 25 mM of L-Glu substantially reduced the phosphorylation of Akt and mTOR compared with the control cells (Fig. 3B), and these reductions were markedly reversed by 3 h of pre-incubation of crocin (Fig. 3B).

Crocin improved cognitive abilities of AD mice, and reduced A β deposition in their brains. In the present study, mice with AD induced by AlCl₃ and D-gal administration were studied to investigate the beneficial effects of crocin in animal models. In the open field test, the AD mice demonstrated significantly more chaotic movements, without purpose, around the central field area compared with the control group (P<0.001; Fig. 4A). The most notable feature of AD is memory loss, with diminishing spatial discernment. The Morris water maze, a useful tool for the evaluation of anti-AD agents, has been widely used to assess learning and memory abilities (26). Herein, in the Morris water maze test, the AD mice required significantly more time to locate the platform hidden in the water, and demonstrated chaotic movements compared with the control group (P<0.05; Fig. 4B). The crocin-treated mice circulated around the periphery significantly more compared with the center in the open field test (P<0.05; Fig. 4A), and required significantly less time to locate the platform in the Morris water maze test (P<0.05; Fig. 4B) compared with the non-treated AD mice. Crocin-alone treatment caused no significant changes to

healthy mice behaviors in the open field (Fig. 4A) and Morris water maze tests (Fig. 4B).

The data obtained from H&E staining demonstrated that crocin displayed no adverse effects on the brains (Fig. 4C), kidneys (Fig. 4D) or spleens of the mice (Fig. 4E).

A β is the principal constituent of neuritic plaques, which have been proposed, mainly on genetic grounds, to be central to the pathogenesis of this form of dementia (27). In the AD mice, significantly lower levels of A β 1-42 in serum and significantly higher levels of A β 1-42 in the cerebral cortex were observed compared with the control (P<0.01; Fig. 5A and B). Comparatively, crocin resulted in a >24.8% increment in the serum levels of A β 1-42 (P<0.05; Fig. 5A), and a >20.8% reduction in the cerebral cortex levels of A β 1-42 compared with the AD untreated mice (P<0.05; Fig. 5B). The results of the immunohistochemical assay revealed that the deposition of A β 1-42 in the hippocampal area was strongly suppressed following 4 weeks of crocin administration (Fig. 5C).

Crocin regulated the levels of cholinergic neurotransmitters in the serum, cerebral cortex and hypothalamus of AD mice. The characteristic memory impairment of AD is closely associated with the lack of cholinergic neurotransmitters (28). Significantly lower levels of Ach and ChAT and higher levels of AchE were observed in the serum, cerebral cortex and hypothalamus of the AD mice compared with the control groups, indicating injury to the cholinergic system in the AD mice (P<0.05; Table I). Four-week crocin administration significantly restored the pathological alterations of the levels of Ach, AchE and ChAT in the serum, cerebral cortex and hypothalamus of the AD mice, suggesting the ability of crocin to mitigate cholinergic dysfunction (P<0.05; Table I).

Crocin regulated the levels of pro- and anti-oxidative factors in the serum, cerebral cortex and hypothalamus of AD mice. The brain is sensitive to oxidative stress, and hyper-levels of ROS may arise in response to β -amyloid precursor proteins and

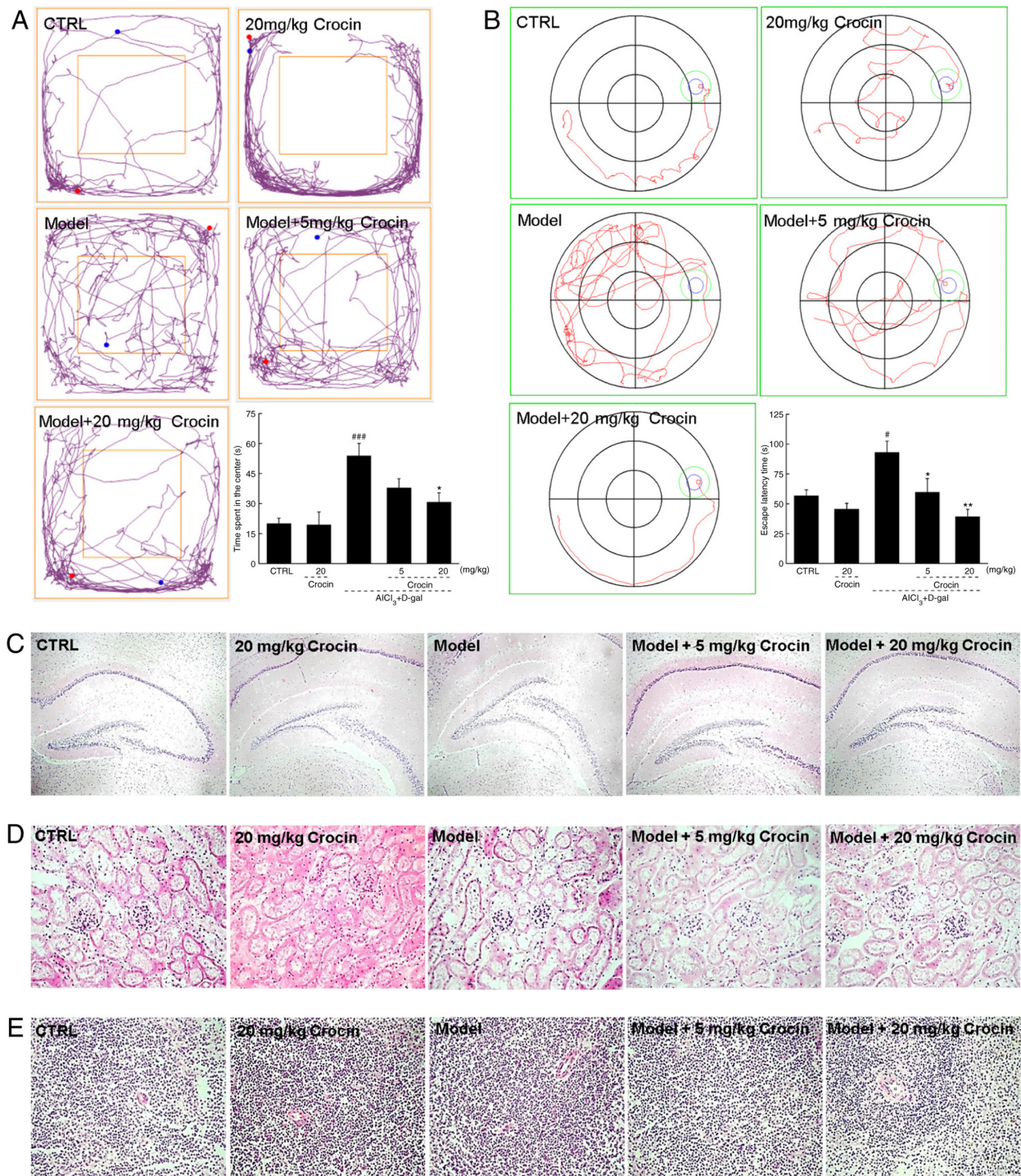


Figure 4. Crocin improves AD-like behaviors in AlCl_3 and D-gal developed AD mice. Compared with non-treated AD mice, crocin administration reduced (A) the time spent in the center of the open-field test, and (B) the time spent locating the platform in Morris water maze. Data expressed as mean \pm the standard error of the mean ($n=20$). [#] $P<0.05$ and ^{###} $P<0.001$ vs. control mice, ^{*} $P<0.05$ and ^{**} $P<0.01$ vs. AD mice. (C) No substantial changes in brain tissue were noted amongst all experimental mice detected by H&E staining (magnification, $\times 40$; $n=10$). No substantial changes in the (D) kidney and (E) spleen were noted amongst all experimental mice detected by H&E staining (magnification, $\times 200$; $n=10$). AD, Alzheimer's disease; AlCl_3 , aluminum trichloride; D-gal, D-galactose; CTRL, control; H&E, haematoxylin and eosin.

mitochondrial DNA, resulting in neuronal apoptosis (29). In the AD-induced mice, the levels of ROS were increased, and the levels of SOD and GSH-Px were decreased significantly in the serum, cerebral cortex and hypothalamus compared with the control mice ($P<0.05$; Table II). Encouragingly, crocin significantly suppressed ROS levels, and enhanced the SOD and GSH-Px levels in the serum, cerebral cortex and hypo-

thalamus of the AD mice compared with the AD alone mice ($P<0.05$; Table II).

Discussion

Although AD is the most prevalent neurodegenerative disease, affecting over 47 million people globally, no

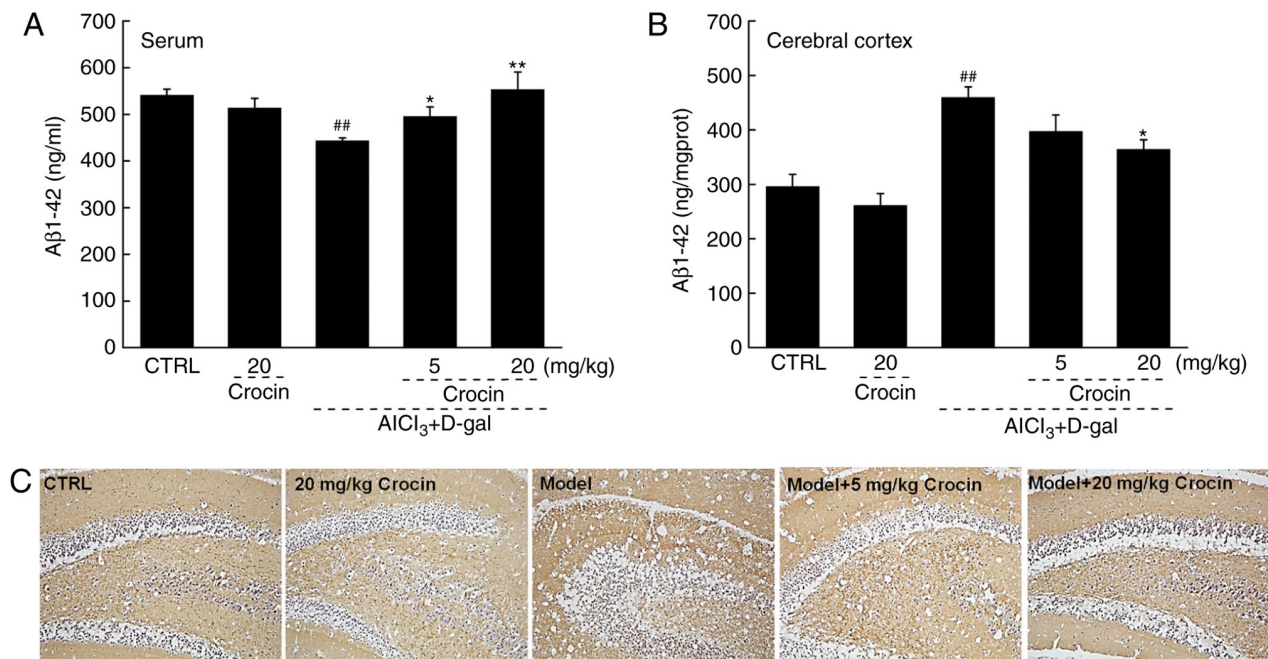


Figure 5. Crocin reduced Aβ1-42 deposition in brains of AlCl₃ and D-gal development AD mice. Compared with non-treated AD mice, 28 days of crocin administration enhanced (A) the content of Aβ1-42 in the serum, and reduced (B) the content of Aβ1-42 in the cerebral cortex analyzing using an enzyme-linked immunosorbent assay. Data expressed as mean ± the standard error of the mean (n=10). ^{##}P<0.01 vs. control mice, ^{*}P<0.05 and ^{**}P<0.01 vs. AD mice. (C) Crocin reduced the deposition of Aβ1-42 in the hippocampus area detected by immunohistochemistry (magnification, x100; n=10). AD, Alzheimer's disease; AlCl₃, aluminum trichloride; D-gal, D-galactose; CTRL, control; Aβ, amyloid β.

satisfactory treatment has been reported so far (30). Neuronal apoptosis is believed to be one of the key events precipitating AD (31). In the present study, crocin mitigated cell apoptosis and mitochondrial dysfunction in HT22 cells induced by L-Glu. A rise in extracellular glutamate concentration may result in neuronal cell death by means of oxidative stress, which is associated with mitochondrial disorder and ROS accumulation (32). Mitochondria are involved in the apoptosis and regulation of intracellular Ca²⁺ homeostasis (33). The overload of intracellular Ca²⁺ results in mitochondrial depolarization, which eventually intensifies oxidative stress-induced cell apoptosis (34,35). ROS, produced by aerobic cells during metabolism, is the foremost cause of oxidative stress (36), resulting in neuronal degeneration and cellular damage in AD (37). Cumulative oxygen damage may cause mitochondrial dysfunction and simultaneously increase ROS production (31). In the present study, crocin inhibited the high expression of cleaved caspase-3, Bad and Bax, and upregulated the low expression of Bcl-xL in L-Glu-exposed HT22 cells. Bcl-2 family proteins are directly responsible for mitochondrial apoptosis, and their ratio is considered to be a biomarker indicating mitochondrial function (37,38). Consistent with a previous study, upregulated Bax and Bad expression accelerates cell apoptosis by increasing mitochondrial permeability; meanwhile, enhanced levels of Bcl-xL help to inhibit cell apoptosis (38). Conversely, MMP dissipation caused by oxidative damage sparks caspase-3, which is cleaved, eventually resulting in apoptosis (39). It has been previously demonstrated that glycyrrhizic acid and *Sparassis crispa* polysaccharides exert neuroprotective effects on differentiated PC12 cells, protecting those cells against the toxicity of

L-Glu by adjusting their MMP levels (40,41). Taken together, the evidence demonstrates that crocin protects HT22 cells against L-Glu-induced apoptosis mainly through modulating mitochondrial apoptotic changes.

Furthermore, crocin upregulated the phosphorylation levels of Akt and mTOR in 24-h L-Glu-exposed cells. Akt serves a central role during apoptosis, and helps to regulate the levels of Bcl-2 family members in AD to exert a neuroprotective effect (30). mTOR, a serine/threonine kinase, is highly conserved in evolution (42), and may be phosphorylated by activated Akt. mTOR is responsible for regulating cell metabolism via the mitochondrial-associated pathway (43). As reported, the activation of Akt signaling may be controlled by ROS levels (44). These data suggest that Akt/mTOR signaling is involved, at least partially, in crocin-mediated neuroprotection of L-Glu-damaged HT22 cells.

Mice with AD, induced by chronic treatment with AlCl₃ and D-gal, exhibited AD-like behaviors in the present study, particularly cognitive disorders and dysmnnesia, which were strongly improved by crocin treatment. The AD-like behaviors associated with brain damage are mainly caused by oxidative stress (45,46), cholinergic system impairment (47,48) and the aggregation of Aβ (49). Abnormally high levels of intracellular ROS accelerate AD progression (29); meanwhile, SOD and GSH-Px, representative endogenous antioxidants, have been reported as the first-line defense against oxidative stress (50). Encouragingly, crocin enhanced the levels of SOD and GSH-Px, and reduced the levels of ROS in the serum, cerebral cortex and hypothalamus of the AD model mice. Aβ plaque deposition in the brain is closely associated with AD (51), and may trigger a series of cascade reactions including mitochondrial dysfunction and oxidative stress (30,37). Aβ is normally cleared from the

Table I. Crocin regulated the levels of Ach, AchE and ChAT in the serum, cerebral cortex and hypothalamus.

	Control	Crocin (20 mg/kg)	Crocin (mg/kg)	
			5	20
Serum				
Ach (μg/ml)	911.9±19.9	1057.0±24.4 ^b	826.3±15.7	1022.5±35.4 ^f
AchE (nmol/l)	138.9±2.2	150.9±6.0	121.8±8.2 ^e	113.2±8.5 ^e
ChAT (pmol/l)	284.7±10.6	283.8±8.6	273.5±9.1 ^d	281.6±2.9 ^e
Cerebral cortex				
Ach (μg/mgprot)	735.7±56.4	741.8±22.1	629.7±61.8	743.2±50.5 ^d
AchE (nmol/gprot)	56.6±4.7	55.9±1.8	97.7±5.4	40.6±2.3 ^f
ChAT (pmol/gprot)	229.3±15.6	207.2±11.3	252.2±25.3 ^d	221.6±1.5
Hypothalamus				
Ach (μg/mgprot)	262.7±13.0	292.0±27.1	210.1±10.2	239.2±16.0 ^d
AchE (nmol/gprot)	53.4±3.1	55.9±1.8	60.4±1.6 ^d	55.7±3.8 ^e
ChAT (pmol/gprot)	144.3±5.4	165.8±6.5	109.6±3.3 ^e	115.0±5.2 ^e

Data are expressed as the mean \pm the standard error of the mean (n=10). ^aP<0.05, ^bP<0.01 and ^cP<0.001 vs. control mice. ^dP<0.05, ^eP<0.01 and ^fP<0.001 vs. AD mice. Ach, acetylcholine; AchE, acetylcholinesterase; ChAT choline acetyltransferase; AlCl₃, aluminum trichloride; D-gal, D-galactose.

Table II. Crocin regulated the levels of ROS, SOD and GSH-Px in the serum, cerebral cortex and hypothalamus.

	Control	Crocin (20 mg/kg)	AlCl ₃ + D-gal	Crocin (mg/kg)	
				5	20
Serum					
ROS (U/ml)	450.8±4.5	475.5±6.3	540.6±5.9 ^b	532.5±6.5	491.3±18.0 ^d
SOD (U/ml)	331.4±7.5	317.5±13.4	277.5±5.0 ^b	309.2±7.7 ^d	336.3±13.7 ^e
GSH-Px (U/ml)	780.0±21.4	756.3±17.7	632.8±20.8 ^b	685±14.9	775±24.4 ^e
Cerebral cortex					
ROS (U/mgprot)	286.3±24.8	323.8±2.1	402.5±23.8 ^c	389.2±35.3	303.3±7.3 ^e
SOD (U/mgprot)	175.3±13.5	162.0±16.8	128.8±5.3 ^a	166.1±13.9 ^d	195.7±19.2 ^e
GSH-Px (U/mgprot)	322.4±20.9	282.2±12.0	269.2±1.9 ^a	358.2±28.0 ^d	412.7±36.5 ^e
Hypothalamus					
ROS (U/mgprot)	126.0±11.8	138.8±8.9	176.6±12.6 ^b	144.2±9.9 ^d	131.9±8.2 ^d
SOD (U/mgprot)	132.7±10.9	118.6±4.3	68.2±3.7 ^c	92.6±6.5 ^e	107.4±9.8 ^e
GSH-Px (U/mgprot)	212.8±20.7	220.1±7.7	121.9±10.2 ^b	199.0±18.6 ^d	211.4±15.9 ^e

Data are expressed as the mean ± the standard error of the mean (n=10). ^aP<0.05, ^bP<0.01 and ^cP<0.001 vs. control mice. ^dP<0.05 and ^eP<0.01 vs. AD mice. ROS, reactive oxygen species; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; AlCl₃, aluminum trichloride; D-gal, D-galactose.

brain into the periphery, while changes in its production or clearance may result in its accumulation in the brain, accompanied by reduced peripheral levels and the clinicopathological manifestations of AD (52). Herein, crocin modulated the concentrations of A β 1-42 in the serum and the brain. Its anti-oxidative activities were key to crocin's ability to improve the cognitive competence of the experimental AD mice.

The central cholinergic system of patients with AD is severely damaged (53). As a major modulator of learning and memory abilities, Ach is dynamically controlled by the terminating enzyme AchE and the synthesizing enzyme ChAT (54). As the level of AchE increases, the cognitive function of the nervous system gradually decreases (28). ChAT is the most suitable factor for monitoring cholinergic neurons (55). Oxidative stress may enhance the expression of AchE (56), which helps to promote the formation of A β and its aggregation into A β plaques (57). The cholinergic system in AD models induced by A β aggregation is severely damaged (58). Altogether, the present data have revealed that crocin regulates the levels of Ach, AchE and ChAT in the serum, cerebral cortex and hypothalamus of AD mice, suggesting the importance of cholinergic function.

The neuroprotective effects of crocin were successfully confirmed in L-Glu-damaged HT22 cells, and the ability of crocin to improve memory abilities and cognitive functions was verified in AlCl₃ and D-gal-induced AD mice. For the first time to the best of our knowledge, the present study verified the roles of oxidative stress-mediated apoptosis and cholinergic functions in the neuroprotective mechanism of crocin.

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Availability of data and materials

All data generated and analyzed during the present study are included in this published article.

Authors' contributions

DW and XCh designed the experiments. CW, XCa, WH, ZL and FK performed the experiments. CW, XCa and WH processed data. DW and CW wrote the paper. DW and XCh revised the paper.

Ethics approval and consent to participate

Institution Animal Ethics Committee of Jilin University approved the experimental protocol (approval no. 20160409).

Patient consent for publication

Not applicable.

Competing interests

The authors have declared that there are no competing interests.

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