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Original article

Safflower yellow reduces lipid peroxidation, neuropathology, tau phosphorylation and ameliorates amyloid β-induced impairment of learning and memory in rats

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ABSTRACT

Insoluble plaques of amyloid β proteins (Aβ) and neurofibrillary tangles of hyperphosphorylated tau are key markers for Alzheimer's disease (AD). Safflower yellow (SY) is one of traditional Chinese medicine extracted from safflower, which is suggested to have therapeutic potential for neurodegenerative disorders. However, whether SY can ameliorate impairment of learning and memory in AD model, and its causal mechanism are still unclear. Here, we applied different doses of SY intragastrically to Wistar rats injected with amyloid β (1–42) for 1 month. By the Morris water maze test, we found that treatment of SY significantly attenuated amyloid β (1–42)-induced impairment of memory in rats. Mechanistically, SY treatment increased the level of superoxidedismutase (SOD) and Glutathione peroxidase (GSH-Px), and decreased the level of malondialdehyde (MDA) and acetylcholinesterase (T-CHE) in brain tissues of AD rats. Pathological analysis also showed that SY treatment inhibited the morphological alteration of neurons and tau hyperphosphorylation induced by amyloid β (1–42)-injection in the cortex and hippocampus. Moreover, SY treatment inhibited CDK-5 and GSK-3 signaling pathways, which are upregulated in AD rats. Our data indicate that safflower yellow can serve as a therapeutic candidate for Alzheimer's disease.

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1. Introduction

As the most common form of dementia, Alzheimer's disease (AD), is a progressive brain disorder affecting brain regions, which are relevant to memory and cognitive functions. The neuropathological hallmarks of Alzheimer's disease are insoluble deposits of amyloid β proteins (Aβ), intracellular neurofibrillary tangles of hyperphosphorylated tau protein, and neuronal apoptosis [1]. Although the pathogenesis of AD is still unclear, multiple evidences have demonstrated that oxidative stress was one of the earliest events in the pathological changes of AD [2]. The increased oxidative stress under AD conditions produces oxidative damage in different regions of the brain including the hippocampus and the cerebral cortex. In addition, excessive generation of oxygen-free radicals or antioxidant deficiency directly results in lipid peroxidation [3] and neuronal apoptosis [4] in AD brain.

Tau is a member of the microtubule-associated protein (MAP) family, which normally serves to stabilize microtubules [5]. It is abnormally phosphorylated at some sites, promoting the formation of paired helical filament-tau and subsequently decreasing microtubule stability, blocking axonal microtubule function, and reducing relevant neuron activity. Hyperphosphorylation of tau can be caused by increasing activity of serine/threonine kinases or decreasing activity of protein phosphatases. Tau can be phosphorylated in vitro by several protein kinases. Among them, cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3β (GSK-3β) are suggested to be the most important candidates that regulate tau phosphorylation in the brain [6]. GSK-3β has the capacity to phosphorylate several MAPs, thus regulating axonal stability through direct interaction with microtubules. Phosphorylated forms of tau and MAP-2 by GSK-3β exhibit decreased affinity toward microtubules and are less stable [7–9]. CDK-5 protein level and enzyme activity have been shown to be increased in AD brains [10]. Therefore, CDK-5 may be involved at the early stage of PHF formation [11]. In addition, CDK-5 and GSK-3 have been shown to promote amyloid β synthesis, and inhibition on GSK-3 was reported to decrease amyloid β levels [12,13]. MAP-

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2 is an abundant cytoskeletal protein predominantly expressed in neurons, which serves as a substrate for most protein kinases and phosphatases present in neurons [8]. Protein phosphatase 2A (PP2A) is responsible for the dephosphorylation of many protein kinases [14]. Furthermore, reduced PP2A activity appears to be a major factor for increasing tau phosphorylation and neurofibrillary tangle pathology [15].

Safflower yellow (SY) is extracted from the plant safflower (*Carthamus tinctorius*) and has been shown to have numerous pharmacological effects. These effects include antiinflammatory, inhibition of hematoblast conglomeration. Our previous studies demonstrated that SY significantly improved cognitive deficits and inhibited oxidative injury in D-galactose/sodium nitrite and scopolamine-induced mice [16,17]. In chronic cerebral hypoperfusion rats, SY also improved learning and memory deficits, inhibited neuronal apotosis and reduced the phosphorylation level of Tau (Ser396) in cortex [18]. In this study we aimed to examine the effect of SY on AD rats injected with amyloid β (1–42), and found that SY ameliorated cognitive impairment and neurodegeneration induced by Aβ. Furthermore, we found that SY decrease the oxidative stress, and tau hyperphosphorylation through inhibiting GSK3β and CDK5 in AD rats. These data suggest that SY may also have potential therapeutic effects in Alzheimer's disease.

2. Material and methods

2.1. Animals and drugs

Male Wistar rats weighing 200–250 g were provided by the Animal Breeding Center of Xinjiang Medical University. Rats were housed 6 per cage and acclimated to standard laboratory conditions (12 h light, 12 h dark cycle) with free access to food and water. All experimental procedures performed were approved by the Institutional Animal Care and Use Committee of National Institute Pharmaceutical Education and Research.

The amyloid β (1–42) (Sigma, USA) was dissolved in sterile saline to a final concentration of 500 μM. The dissolved amyloid β (1–42) was then incubated at 37 °C for 7 days to obtain aggregated amyloid β (1–42). Safflower yellow was purchased from Yunnan Tonghai Yangshi Biotechnology Co., Ltd having total yellow pigment >99% [19]. Safflower yellow was dissolved in distilled water at concentrations of 10, 30, and 100 mg/kg. The dosage was calculated by the weight of the rats. Galanthamine hydrobromide was purchased from the National Institutes for Food and Drug Control, and the purity of the compound was 100%.

2.2. Alzheimer's disease model

Wistar rats were anesthetized with 3.5 ml/kg chloral hydrate and placed in a stereotaxic instrument. Body temperature was maintained at 37 °C using an electrical heating pad. The aggregated amyloid β (1–42) was injected directly into the bilateral rat hippocampus with a 2.5 ml CMA microsyringe, at coordinates determined from the Paxinos and Watson rat brain atlas. Holes were drilled in the skull over the lateral ventricle using the following coordinates: 3.8 mm posterior to bregma, ±2.5 mm lateral, and 3.5 mm below the dura. Amyloid β (1–42) was injected using stereotaxic instruments and the injection rate was approximately 0.5 μl/min. Sham animals were injected in an identical manner with the same amount of sterile saline. The animals were taken to their individual cages after the surgery.

Ninety Wistar rats were randomly assigned to 6 groups: saline-sham, amyloid β (1–42)-model, amyloid β (1–42)-safflower yellow (10 mg/kg)-treated, amyloid β (1–42)-safflower yellow (30 mg/kg)-treated, amyloid β (1–42)-safflower yellow (100 mg/kg)-treated, and amyloid β (1–42)-galanthamine (3 mg/kg)-treated groups. Starting from the fifth day after surgery, drugs were administered by oral gavage once a day continuously for weeks to each group of rats. Changes in learning and memory were then investigated using the Morris water maze.

2.3. Morris water maze

The Morris water maze was a round pool with a diameter of 150 cm and height of 50 cm divided into 4 quadrants labeled I, II, III, and IV. A cylindrical platform 10 cm in height and 15 cm in diameter was placed 1.5 cm below the water surface in the middle of quadrant III. Rats were placed into the water at the midpoint of the wall in each quadrant.

Two test methods were used. The first was the positioning navigation test, which began when the rats were placed in the water and ended when they located the platform and climbed onto it. The duration of this test was recorded as the full-time average escape latency. The rat had to stay on the platform for 2 s for it to be considered to have found the platform. If rats did not find the platform within 60 s, they were led to the platform and left there for 10 s to familiarize with the environment and platform position. For these trials, 60 s was recorded as the escape latency. The second test was the spatial exploration test. On the fifth day, a probe trial was given for memory retention by removing the platform from the pool and allowing each rat to swim freely for 60 s. During this test, the number of crossings of the former platform location was recorded.

2.4. Anti-oxidative enzyme activity

After the Morris water maze experiments, the animals were decapitated and the hippocampus and cortex were quickly removed, rinsed with saline, and frozen (−80 °C). The brain samples were rapidly homogenized of pre-cooled physiological saline and then centrifuged at 3500 rpm for 15 min at 4 °C. The supernatant obtained was used to measure antioxidative enzyme activity including superoxide dismutase, malondialdehyde, and acetylcholinesterase using assay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.5. Nissl staining

After the Morris water maze experiments, the rats were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.) and perfused trans-cardially with 100 ml of saline followed by 400 ml of 4% paraformaldehyde. The brain was dissected and stored in 4% paraformaldehyde, before being dehydrated by the conventional alcohol gradient, embedded in paraffin, and cut into 4-μm-thick coronal slices. Consecutive slices were mounted on clean slides treated by poly-L-lysine, incubated in a 60 °C oven for 2 h, and saved for later use. For Nissl staining, the sections were placed in xylene to dewax and alcohol for benzene removal. After washing in distilled water, the sections were stained in 1% toluidine blue solution preheated to 50 °C and incubated for 20 min at 56 °C. The sections were then soaked in 70% alcohol for 1 min and viewed carefully under a microscope while they were differentiated in 95% alcohol. Finally, they were dehydrated in 100% alcohol immediately after Nissl bodies appeared and then sealed with neutral balsam.

2.6. Immunohistochemistry

We used the Envision method and 3, 3′-diaminobenzidine (DAB) immunohistochemistry staining to detect the expression of MAP-2 proteins in the hippocampus. Slices were first incubated in a 60 °C oven for 4 h and were routinely dewaxed and hydrated. They were placed in a staining dish containing citrate buffer

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