RhoA/Rock2/Limk1/cofilin1 pathway is involved in attenuation of neuronal dendritic spine loss by paeonol in the frontal cortex of D-galactose and aluminum-induced Alzheimer’s disease-like rat model

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INTRODUCTION

Dementia is one of the major causes of disability and dependency among older people worldwide. Every year, there are nearly 10 million new cases (WHO, 2019). Alzheimer disease (AD) is the most common form of dementia and may account for 60–70% of cases (Tang et al., 2016). The early clinical symptoms of the disease are mainly cognitive impairment such as memory and visuo-spatial skills, and then gradually worsen, accompanied by emotional behavioral reactions such as depression, anxiety, and decreased activity (Lyketsos et al., 2011; Ameen-Ali et al., 2017; Götz et al., 2018). The typical histopathological hallmarks of AD are the aggregation of amyloid-beta in extracellular senile plaques, accumulation of tau in intracellular neurofibrillary tangles, and neuron loss, in the brain especially in hippocampus and frontal cortex (Hyman et al., 2012; Grontvedt et al., 2018; Alzheimer’s Association, 2019). However, the etiology and pathogenesis of AD are un-
A number of studies have revealed that AD undergoes a progressive lesion process and is thought to begin 20 years or more before symptoms arise, with small changes in the brain (Jack et al., 2009; Heiko et al., 2011; Bateman et al., 2012; Reiman et al., 2012; Villemagne et al., 2013; Gordon et al., 2018). Only the brain synapses and neurons involved in thinking, learning, and memory (cognitive function) have been damaged or destroyed, and AD shows symptoms (Alzheimer’s Association, 2019). Clinical histopathological studies have demonstrated that in layer II and III pyramidal neuron dendrites, spine density is similar among controls and CAD cases (cognitively normal individuals with high AD pathology) but is reduced significantly in AD dementia cases, suggesting a close relationship between symptoms and spines, the postsynaptic component (Boros et al., 2017). Accordingly, it is proposed that pathology characterized by Aβ and hyperphosphorylated-tau occurs in early AD, but does not cause significant damage and loss of synapses and neurons, and therefore does not exhibit AD symptoms; but over time, Aβ synergizes with hyperphosphorylated-tau, causing significant dendrite and synaptic loss, which in turn shows symptoms of AD (Ittner and Ittner, 2018; Alzheimer’s Association, 2019).

Given the pathogenesis of AD remains to be illustrated, there is currently no drug approved to halt or delay the progression of AD (Anderson et al., 2017). With the failure of phase 3 clinical trials for the treatment of AD drugs in recent years, including the β secretase inhibitor verubecestat (Egan et al., 2018) and the anti-Aβ drugs semagacestat (Doody et al., 2013), bapineuzumab (Vandenberghe et al., 2016) and solanezumab (Neurology, 2017), it is even more important to explore new targets and drugs.

In China, polyphenolic Chinese herbal medicine has been used for thousands of years in clinical application, and no toxic effects have been reported (Zhang et al., 2019). Polyphenols are usually easily absorbed by the gastrointestinal tract and quickly cross the blood-brain barrier (Lin, 2011). Paeonol (Pae, 20-hydroxy-40-methoxyacetophenone), a simple phenolic compound, is the main active component extracted from Moutan cortex and Cynanchum paniculatum. A lines of studies have shown that paeonol has various biological and pharmacological activities including anti-inflammation (Tz-Chong et al., 2003; Ishiguro et al., 2006), antioxidation (Liu et al., 2017), anti-neurotoxicity (Liu et al., 2018), anti-tumor effects (Hagen et al., 2003; Kubes and Mehal, 2012), and anti-anxiety (Ishiguro et al., 2006), as well as anti-diabetic effects (Lau et al., 2007), anti-hyperglycemic effects (Juan et al., 2010) and protection of rat heart (Ma et al., 2016).

The latest researches reveal that paeonol can inhibit oxidized low-density lipoprotein-induced vascular endothelial cells autophagy by upregulating the expression of miRNA-30a (Li et al., 2018a), ameliorate diabetic renal fibrosis through promoting the activation of the Nrf2-ARE pathway via up-regulating sirt1 (Zhang et al., 2018), attenuate the inflammatory responses of endothelial cells via stimulating monocytes-derived exosomal microRNA-22 (Liu et al., 2018), and ameliorate glucose and lipid metabolism in experimental diabetes by activating Akt (Xu et al., 2019). In addition, paeonol has an anti-atherosclerotic effect against vascular smooth muscle cell proliferation by up-regulation of autophagy via the AMPK-mTOR signaling pathway (Wu et al., 2017), prevents bleomycin-induced pulmonary inflammation and fibrosis in mice through the inhibition of the MAPKs/Smad3 signaling (Liu et al., 2017), and alleviate nerve damage caused by intracerebral hemorrhage by activating the PI3K/AKT pathway (Li et al., 2018b). Our previous study shows that paeonol can attenuate D-galactose and aluminum-induced dendritic damage in the hippocampus (Han et al., 2017). In this study, we found involvement of Rho/Rock2/Limk1/cofilin1 pathway in paeonol’s attenuation of neuronal dendritic loss in the frontal cortex of D-galactose and aluminum-induced rat model of Alzheimer’s disease.

METHODS

Reagents and Drugs

The chemical structure of paeonol (C13H14NaO8S, a derivative of paeonol) was shown in Fig. 1. Paeonol was shown to have the same pharmacological effects as paeonol and has been approved by the Chinese FDA for clinical use in the treatment of muscle pain, arthralgia, rheumatism, neuralgia and abdominal pain (No.H20064790, http://app1.sfda.gov.cn/datasearchcnda/face3/dir.html), was purchased from Jinling Pharmaceutical Company (Nanjing, China). D-galactose (D-gal) was purchased from
Sangon Biotech (Shanghai, China). Aluminum (AlCl₃) was purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). Phospho-Tau (Ser202) and Aβ₁₋₄₂ antibodies (used to recognize the Aβ fibrils) were purchased from Bios Biological Technology Co., Ltd (Beijing, China). RHOA antibody was purchased from Sangon Biological Technology Co., Ltd (Shanghai, China). LIMK1, ROCK2 and PI3K antibodies were purchased from Boster Biological Technology Co., Ltd (Wuhan, China). A11 antibody (used to recognize the Aβ oligomers, SAB5200113) was purchased from Sigma-Aldrich (St. Louis, USA). Cofilin1, SSH1 antibodies and DAPI were purchased from Santa Cruz Biotechnology. Other chemicals were purchased from Sigma.

Animals and Treatments

Adult male Sprague-Dawley (SD) rats (180–200 g) were obtained from the Shandong Experimental Animal Center (license number, SCXK20140007; Jinan, China) and housed at 20 ± 2°C, with a light cycle between 08:00 and 20:00 h. Food and water were provided ad libitum. Animals were treated according to the Guidelines of the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People’s Republic of China on November 14, 1988. All animal experiments were conducted with the approval of the Animal Use and Care Committee of Anhui Normal University (2015002).

AlCl₃ and D-gal co-induced animal models may be used to characterize AD-like behavioral and pathological features, as well as pathologic processes without the genetic background of gene mutations and have thus been widely used in AD-related studies (Sun et al., 2009; Lu et al., 2017; Wei et al., 2017; Xiao et al., 2017; Chiroma et al., 2019). In this study, D-gal was dissolved in physiologic saline (0.9%) and injected (100 mg/kg/day, once per day, s.c.). AlCl₃ was dissolved in double-distilled water and administered (40 mg/kg/day, once per day, i.g.). Model induction lasted for 42 days.

Following a one-week adaptation period, thirty male rats were randomly divided into 3 groups of 10 rats each: the control group (CON), D-gal + AlCl₃ group (DGA group treated with D-gal + AlCl₃ for 42 days), Paeonolsilatate sodium treatment group (PEA group treated with 50 mg/kg, i.p., 1 h before D-gal + AlCl₃ for 42 days). The control treatments were volume-matched vehicle.

Behavioral tests

Rats were brought to the testing room 30 min before the start of each behavioral test and remained in the same room through the test. All behavioral tests were performed during the light cycle under lighting condition in 70 lx by experimenters blind to treatment information.

Open field test (OFT)

The OFT is a well-established paradigm to examine fear in rodents (Walsh and Cummins, 1976). The test apparatus was made of opaque materials into lidless boxes with length, width and height of 80 cm × 80 cm × 40 cm respectively. The bottom was divided into 25 quadrates of 16 cm × 16 cm by a white line. First, the rats were placed in the laboratory 30 min prior to testing to adapt to the environment. And then, each rat was placed in the center area, which was defined as 9 squares in the center, and the behaviors were recorded for 5 min in a quiet environment. The number of squares crossed, number of clean movements, and number of rears were recorded. The observers were blind to the rats of the different treatment groups. The device was cleaned with 70% ethanol thoroughly after each trial.

Elevated plus maze test (EPMT)

The EPMT was commonly used to assess anxiety behaviors (Pellow et al., 1985). The high cross apparatus consisted of four black arms (50 cm × 10 cm) and a central square platform (10 cm × 10 cm). Two of the arms were closed arms with walls (40 cm height), called the closed arms. And the other two arms were open without walls, called the open arms. The maze was elevated 50 cm from the floor. There was a camera right above it. The animals were placed individually at the center of the maze, facing one of the open arms, and their activities were recorded for 5 min. When a rat placed its four paws into an arm, it was counted as entry in the arm. Recordings were made of the time spent in the open arms, the time spent in the closed arms, and the number of head dipping. The maze was cleaned with 70% ethanol thoroughly after each trial. The experimenters who recorded the data were unaware of the grouping.

Morris water maze test (MWMT)

The MWMT, which was usually used to assess spatial learning and memory capability, was carried out according to methods described previously (Morris, 1984; Vorhees and Williams, 2006). The pool (1.5 m in diameter, 50 cm in height) was filled with water to a depth of 30 cm. A circular escape platform (15 cm in diameter) was submerged 2 cm below the water surface. The
water was clouded with black food coloring to prevent the visual image of the submerged platform. Rats were trained to find a hidden platform in a water maze for five consecutive days. For each trial, the rat was placed facing the wall at one of four designated start points. Each rat was allowed to find the platform for 60 s and remained on the platform for 20 s. If he could not reach the platform within 60 s, he was guided gently to the platform and escape latency (time taken to find the platform) was recorded as 60 s. The rat stayed on the platform for 20 s. The average escape latencies of the four trials per day were used for statistical analysis. A longer time spent in finding the platform was used to assess the extent of learning impairment.

A probe trial without the platform was performed twenty-four hours after the final acquisition trial. During probe trials, the animals were allowed to swim for 60 s within the pool while their time spent in the target quadrant was recorded. The longer a rat stayed in the target quadrant, the better it scored for spatial memory.

The swimming behaviors of each rat were monitored via a television camera mounted overhead. The time was manually recorded with a stopwatch by two observers. Modeling, treatment and behavioral test schedule were shown in Fig. 2.

**Tissue preparation**

Twenty-four hours after the behavioral tests, the rats were deeply anesthetized with 1% carbutal and perfused (via a transcardial approach) with 0.9% saline. And then, rats were treated according to the requirements of different experimental methods. Six rats of each group were euthanasia via decapitation and the half frontal cortex tissue of which was stored in Golgi-cox solution, while the other half of which was stored in a -80°C refrigerator for further Western blot analysis. Four rats in each group, perfused with 4% paraformaldehyde and euthanasia, followed by the frontal cortex tissues being collected and embedded in paraffin wax. The paraffin-embedded tissues were cut with a Leica microtome (RM2235) into serial coronal sections (6 µm).

**Golgi-Cox staining**

The Golgi-Cox staining was mainly based on our previous papers (Han et al., 2017; Zhu et al., 2018). Briefly, frontal cortex tissues were stored in Golgi-Cox solution in the dark at 37°C for 48 h and subsequently sectioned (200 µm thick coronal sections) using a vibratome (NVSL/NVSLM1). The sections were then immersed in alcohol (50%) for 5 min, ammonium hydroxide for 5±10 min, and 5% sodium thiosulfate in the dark for 10 min and serially dehydrated in solute alcohol, including 70%, 80%, and 95% alcohol (7 min × 2 for each) and 99% 1-butanol for 7 min; the samples were subsequently cleaned in xylene for 5 min and medium mounted with coverslips using Rhamsan gum.

**Immunohistochemistry**

Coronal sections (6 µm) of the brain samples were rinsed with PBS over 5 min and subsequently blocked

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**Fig. 2.** Schematic diagram of the timeline for establishing an AD model, treatment and behavioral test. The upper yellow rectangular box indicated adaptation treatment from day 0 to day 7. The upper purple rectangular box or down blue rectangular box indicated the treatment with D-gal and AlCl3 or Paeononilsilatie sodium from day 8 to day 49, respectively. Paeononilsilatie sodium was given 1 h before D-gal and AlCl3 treatment. The downward or upward arrows indicated the rats performed for the behavioral test. OFT, open field test; EPMT, elevated plus maze test; MWMT, Morris water maze test. The rats were sacrificed on day 57.
for 30 min at room temperature in PBS-Triton (0.1 M PBS and 0.3% Triton X 100). After blocking, samples were subsequently treated twice (2 × 10 min) by microwaves (700 W) in 0.05 M citrate buffered saline (pH 6.0). After being washed in PBS, the samples were blocked with normal bovine serum in PBS for 1 h at 37°C, followed by incubation with primary antibodies (rabbit Aβ1-42 pA (1:200), rabbit phospho-tau (p-tau) pA (1:200) or mouse cofilin1 mA (1:200) overnight at 4°C. Single labeling with DAB (Aβ1-42 and p-tau immunoreactivity); after being washed, the sections were incubated with the secondary antibody goat anti-rabbit (Boster Biotech, Wuhan, China) for 1 h at 37°C. Double labeling with fluorescein labeling methods previously described by our group (Ya-Ping et al., 2003). Sections were then washed over 5 min two times with PBS and incubated with the secondary antibody biotinylated horse anti-mouse IgG diluted in PBS (1:200) that contained 5% normal horse serum for 1 h at 37°C, which was performed as previously described (Hu et al., 2003; Han et al., 2017). Then these sections were incubated with CyTM3-labeled streptavidin (Kirkegaard and Perry Laboratories) diluted in PBS for 30 min at room temperature, followed by incubation with 0.05 M glycine-HCl buffer saline (pH 2.2) for 2 h at room temperature to quench additional antibodies; the samples were subsequently incubated with second primary antibodies against rabbit p-cofilin1 pA (1:200) for 24 h at 4°C and incubated with the fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h, diluted in PBS (1:100). Then sections incubation with DAPI for 10 min. Finally, the sections were cover slipped with glycerin. Control samples were simultaneously performed following the same procedures as the test samples with the exception that the primary antibodies were omitted.

Images were obtained with an Olympus FV1000 laser scanning confocal microscope. FV10-ASW 4.2 Viewer software was used for image format conversion. To assess the distribution of cofilin1 in neurons, plots of fluorescence intensity versus distance were generated using the RGB profile plot function (intensity line profile for red channel) of ImageJ 1.49 s (NIH).

Western blotting

Briefly, 10% (w/v) tissue homogenates were centrifuged at 12,000×g for 30 min at 4°C, and the supernatants were transferred to clean tubes and stored. Protein concentration was determined using the bicinchoninic acid assay. The proteins (40 μg of total protein) were electrophoresed on various concentrations of Tris-tricine polyacrylamide gels (under non reducing conditions) and transferred onto PVDF membranes. The blots were blocked with 5% nonfat dry milk in Tris-buffered saline Tween 20 (TBS-T) for 2 h at room temperature. Then, membranes were incubated with antibodies (Table I) were diluted in 0.1% BSA/TBS-T, covered with plastic wrap and incubated for 12 h at 4°C. Bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies and the ECL detection system (BHEYOTIME Biological Technology Co., Ltd, China). Densitometric analysis of antibody specific bands was performed with NIH ImageJ version 1.34 software.

Image analysis

Image analysis and quantification of the histological sections were performed by one author who was not aware of the experimental information. Images were captured under Laser scanning confocal microscope (OLYMPUS FV1000) and analyzed using image analysis software (ImageJ). In serial sections between bregma +2.22 mm and +1.98 mm, one section was selected at intervals of every sixth section. In frontal cortex, three fields of view (×60 objective) were randomly selected for accounting the number of p-tau immunopositive cells and the percentage of the field of view covered by Aβ1-42. In frontal cortex layer III, three fields of view (×60 objective) each region (in the basal dendritic region and the apical dendritic region of pyramidal cells) were randomly selected for the further statistics in the percentage of the field of view covered by fibrillary actin as described previously (Han et al., 2017).

Table I. Antibodies and dilution ratio.

| Antibody                        | Dilution ratio |
|---------------------------------|----------------|
| p-cofilin1 (Ser3)               | 1:1000         |
| Cofilin1                        | 1:500          |
| SSH1                            | 1:400          |
| RHOA                            | 1:500          |
| LIMK1                           | 1:300          |
| ROCK2                           | 1:250          |
| PI3K                            | 1:300          |
| GAPDH                           | 1:400          |
| HRP-conjugated Goat Anti-Rabbit IgG | 1:5000   |
| HRP-conjugated Goat Anti Mouse IgG | 1:4000   |
| H&L conjugated Goat Anti-Rabbit IgG | 1:1000  |
Quantification of Dendrites and Dendritic Spines

Measurements of the dendritic branch and length were based primarily on previous literature (Mavroutidis et al., 2011; Lazcano et al., 2014). Briefly, the regions tested were determined at a low magnification according to the description by Paxinos and Watson (2006), and more than 5 neurons in the frontal cortex layer III per mouse were selected to acquire their photographs, which were used for quantificational analysis. The criteria used to select neurons for quantitative analysis have been previously described (Flores et al., 2005; Bringas et al., 2013; Lazcano et al., 2014). Each selected neuron was analyzed using ImageJ software. Neuronal branches were traced by the NeuronJ plug-in to count their total dendritic length, and dendritic intersections that cross the concentric circles were counted using the Sholl analysis plug-in protocol (Kolb et al., 1998; Flores et al., 2005; Martinez-Tellez et al., 2011; Lazcano et al., 2014). Briefly, the analysis was divided into three steps: step 1, on our previous literature (Han et al., 2017; Zhu et al., 2013); step 2,24 = 39.741, \( P < 0.001 \); PAE vs. DGA, \( P < 0.001 \) (Fig. 3B) in OPT, as well as reduction in the number of squares crossed (\( F_{2,24} = 39.741, P < 0.001 \); post hoc comparisons: DGA vs. CON, \( P < 0.001 \); PAE vs. DGA, \( P < 0.001 \)) (Fig. 3A) and the number of rearing movements (\( F_{2,24} = 32.627, P < 0.001 \); post hoc comparisons: DGA vs. CON, \( P < 0.001 \); PAE vs. DGA, \( P < 0.001 \)) (Fig. 3B) in OPT, as well as reduction in the time spent open arms (\( F_{2,24} = 39.741, P < 0.001 \); post hoc comparisons: DGA vs. CON, \( P < 0.001 \); PAE vs. DGA, \( P < 0.001 \)) (Fig. 3C) and increase in the time spent in closed arms (\( F_{2,24} = 39.741, P < 0.001 \); post hoc comparisons: DGA vs. CON, \( P < 0.001 \); PAE vs. DGA, \( P < 0.001 \)) (Fig. 3D) in EPMT.

In MWMT, Pillai’s trace of the repeated ANOVA showed that there was no significant difference between day and group (\( F_{2,24} = 2.149, P = 0.05 \)). Tests of between-subjects effects displayed a significant difference between groups in the escape latency (\( F_{2,24} = 6.120, P = 0.01 \)). One-way ANOVA revealed differences between groups found on the day 4 (\( F_{2,24} = 7.563, P = 0.01 \)) and 5 (\( F_{2,24} = 13.562, P = 0.001 \)). Pairwise comparisons demonstrated that paeonol decreased D-galactose and aluminum-induced increase in the escape latency on the fourth day (DGA vs. CON, \( P = 0.001 \); PAE vs. DGA, \( P = 0.01 \)) (Fig. 3E). The probe trial performances were assessed by removing the platform 24 h after the acquisition trials. The time spent in the region of target quadrant

| Type               | Parameters                     |
|--------------------|--------------------------------|
| Mushroom spines    | Mean Width (head) > 0.6 μm     |
| Stubby spines      | Length : Width Ratio < 1       |
| Filopodia spines   | Length > 0.6 μm                |

Table II. Classification of spine morphology using RECONSTRUCT software.
around the platform showed a significant difference between the groups ($F_{2,24}=9.848$, $P<0.01$). Post hoc comparisons indicated that $D$-galactose and aluminum induced a marked reduction in the time spent in the area around the platform ($P<0.001$), which can be attenuated by treatment with paeonol ($P<0.01$) (Fig. 3F).

![Fig. 3. Effects of paeonol on the behavioral performances in OFT (A, B), EPMT (C, D) and MWMT (E, F). Paeonol treatment (50 mg/kg, i.p.) attenuated the $D$-gal and AlCl$_3$-induced decrease in the number of squares crossed (A) and the number of rearing movements (B) in the OFT. In the EPMT, $D$-gal and AlCl$_3$ induced a decrease in the time spent in open arms (C) and an increase in the time spent in closed arms (D), these of which were significantly improved by paeonol treatment. (E) The average escape latencies of four acquisition trials per day in DGA rats from days 4 to 5 were significantly more than those in CON rats. Paeonol pretreatment produced significant protective effects, particularly at days 4~5. (F) Probe trials 24 h after acquisition trials indicated that animals treated with paeonol spent more time in the target quadrant than animals in DGA. Data expressed as the means ± SEM (n=8-10). **$P<0.01$, ***$P<0.001$ versus CON; *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus DGA.]
Paeonol decreased D-galactose and aluminum-induced increase of oligomeric Aβ and hyperphosphorylated-tau in the frontal cortex

Immunohistochemical examination showed that paeonol significantly attenuated D-galactose and aluminum-induced increase of oligomeric Aβ (Fig. 4A a, b, c) and hyperphosphorylated-tau (Fig. 4B a, b, c) in the frontal cortex. Two-tailed t test indicated that the percentage of Aβ immunopositive area in the visual field area ($P<0.001$) (Fig. 4A d) and the hyperphosphorylated-tau immunopositive cell number per visual field area ($P<0.001$) (Fig. 4B d) in PAE were markedly lower than that in the DGA.

Western blotting revealed that D-galactose and aluminum caused a noteworthy increase in oligomeric Aβ levels, which were downregulated by treatment with paeonol (Fig. 4C). Numerous studies have demonstrated that some types of Aβ oligomers have strong neurotoxicity, such as ~32 KD Aβ oligomers (Fernando et al., 2010; Acta Neurobiol Exp 2020, 80: 225–244).

Fig. 4. Effects of Paeonol on D-gal and AlCl₃-induced Aβ and tau burdens in the frontal cortex. (A) Grayscale microscope pictures in immunohistochemical staining with Aβ1-42 antibody in the frontal cortex in CON (a), DGA (b), and PAE (c); administration of paeonol (50 mg/kg, i.p.) for 6 weeks significantly reduced the percentage area of Aβ1-42-positive deposits (d). (B) Phospho-tau immunohistochmical staining (p-Ser 202) in the frontal cortex in CON (a), DGA (b), PAE (c), and their statistical analysis in the number of p-tau positive cells (d). (C) Western blot bands with A11 antibody identifying different molecular weights of Aβ oligomers in the frontal cortex in CON (left column), DGA (middle left column), PAE (middle right column). Paeonol did not affect the expression of 16 KD Aβ oligomers (D); however, it decreased the expression of 28 KD (E), 32 KD (F), and 56 KD Aβ oligomers (G). Data expressed as the means ± SEM (n=4~5). **$P<0.01$, ***$P<0.001$ versus DGA. Scales: 50 μm in A (c) and B (c).
Yang et al., 2011; Washington et al., 2014), especially ~56 KD Aβ oligomers (Lesne et al., 2013). Therefore, we examined the effects of paeonol on the expression levels of 16 KD, 28 KD, 32 KD, and 56 KD. Two-tailed t test showed that paeonol treatment remarkably reduced Aβ oligomer levels in 28 KD ($P<0.01$), 32 KD ($P<0.01$), and 56 KD ($P<0.001$), but not in 16 KD ($P>0.05$).

**Paeonol relieved D-galactose and aluminum-induced dendrite and dendritic spine loss in the frontal cortex**

Excitatory synapses occur on dendritic protrusions called dendritic spines, and synapse activity is inseparably linked to spine density and morphology (Yasun-

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**Fig. 5. Effects of Paeonol on the dendritic length and branches in frontal cortex.** (A) Frontal cortex pyramidal neurons and their traces drawn by ImageJ software. Paeonol treatment (50 mg/kg, i.p.) for 6 weeks significantly increased the number of dendritic branches (B), extended the length of basal dendrites (C), and increased the length of apical dendrites (D). Data expressed as the means ± SEM (n=5). *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus CON; *$P<0.05$, **$P<0.01$ versus DGA.
Dendritic spine and neuronal synapse loss correlate more strongly with cognitive impairment than classical pathologic markers of AD (Dekosky and Scheff, 1990; Terry et al., 2010). So, in this study, we evaluated the effects of paenol on the dendrite and dendritic spine loss induced by D-galactose and aluminum in the frontal cortex.

As shown in Fig. 5A, the dendrites were traced for counting their length by the NeuronJ plug-in, and the dendritic intersections that cross the concentric circles were counted for the dendritic branches by the Sholl analysis plug-in protocol. One-way ANOVA indicated that significant differences of dendritic length between groups were found in the basal dendrites \((F_{2,14} = 19.085, P<0.001)\) and apical dendrites \((F_{2,14} = 15.781, P<0.001)\). Post hoc comparisons showed that there were remarkable differences between CON and DGA \((P<0.001)\) and between DGA and PAE \((P<0.01)\) in the basal dendrites (Fig. 5C), as well as between CON and DGA \((P<0.001)\) and between DGA and PAE \((P<0.01)\) in the apical dendrites (Fig. 5D). A repeated ANOVA revealed that the total dendritic intersections between groups existed dramatically different \((F_{2,14} = 9.714, P<0.001)\; \text{post hoc comparisons: CON vs. DGA, } P<0.001; \text{PAE vs. CON, } P<0.05; \text{PAE vs. DGA, } P<0.05)\). Segmentation statistics showed that the significant differences between the groups were found at some segments, especially at segment 3 \((F_{2,14} = 4.702, P<0.05; \text{post hoc comparisons: CON vs. DGA, } P=0.01; \text{PAE vs. DGA, } P<0.05)\), 4 \((F_{2,14} = 4.608, P<0.05; \text{post hoc comparisons: CON vs. DGA, } P<0.01; \text{PAE vs. DGA, } P<0.05)\), and 5 \((F_{2,14} = 4.467, P<0.05; \text{post hoc comparisons: CON vs. DGA, } P<0.01; \text{PAE vs. DGA, } P<0.05)\).

Fig. 6B (a) and B (b) represent the slice view of dendritic segments in Fig. 6A (a) (basal dendrites) and A (b) (apical dendrites), respectively. Visually, the differences in the dendritic morphology and density can be found between the groups. One-way ANOVA revealed the significant differences of dendritic spine density between the groups detected in both basal dendrites \((F_{2,14} = 9.948, P<0.01)\) and apical dendrites \((F_{2,14} = 4.461, P<0.05)\). Post hoc comparisons indicated that in the basal and apical dendrites, D-galactose and aluminum resulted in a remarkable decrease of the spine density, which can be relieved by treatment with paenol in basal dendrites (Fig. 6C).

**Fig. 6.** Effects of paenol on the density and type of dendritic spines in frontal cortex. (A) Pyramidal neurons in the frontal cortexlayer IIIstained by Golgi-Cox (scale: 20 μm). (B) Slice view acquired by Laser scanning confocal microscope (FV1000, 60×6 for objective magnification) at the apical (left column) and basal (right column) dendritic segments stained by Golgi-Cox method in frontal cortex. Paenol treatment for 6 weeks significantly increased the dendritic spine density in apical dendrites but not in basal dendrites (C). (D) Types of dendritic spines (scale: 2 μm). In basal dendrites, a difference between CON and DGA was only found in the mushroom-type dendritic spines (E). However, in the apical dendrites, significant differences between groups existed in the mushroom-and filopodia-type spines (F). Data expressed as the means ± SEM. *P<0.05, **P<0.01, ***P<0.001 versus CON; #P<0.05 versus DGA.
In this study, according to the morphology of dendritic spines, they were divided into three types, mushroom, stubby and filopodia (Fig. 6D). In the basal dendrites, only mushroom-type dendritic spines existed differences between groups ($F_{2,14}=5.383$, $P<0.05$). Post hoc comparisons only showed a significant difference between CON and DGA ($P<0.05$), not between CON and PAE ($P>0.05$) or between PAE and DGA ($P>0.05$) (Fig. 6E). However, in the apical dendrites, significant differences between groups were observed in the types of mushroom ($F_{2,14}=8.901$, $P<0.01$) and filopodia ($F_{2,14}=4.132$, $P<0.05$). Post hoc comparisons manifested that paeonol treatment markedly attenuated D-galactose and aluminum-induced decline in the density of mushroom-type spines (CON vs. DGA, $P<0.01$; PAE vs. DGA, $P<0.05$) and filopodia-type spines (CON vs. DGA, $P<0.05$; PAE vs. DGA, $P<0.05$). Interestingly, there were no differences in the percentage of any types of dendritic spine found between the groups either in the basal dendrites or in the apical dendrites.

![Fig. 7](Image)

**Fig. 7.** Effects of Paeonol on F-actin density labeled by phalloidin staining in the frontal cortex. (A) A schematic diagram of the statistics area in the frontal cortex. MAP2 immunoreactivity (red) was used to distinguish the different areas (basal dendrite area and apical dendrite area) in the frontal cortex. (B) Magnification of the statistics area in a single channel of 488 nm (OLYMPUS FV1000). (C) Image of (B) was processed using ImageJ software. The background was subtracted with a rolling value of 15, converted to 8-bit deep images and binarized using a determined threshold value (reduce noise 5, particles 2~`). Statistical analysis for the percentage of phalloidin immunopositive area in basal dendrite area or apical dendrite area was expressed in (D) and (E), respectively. Data expressed as the means ± SEM (n=4~5). Scale bar in (A) represents 20 μm and in (B) represents 5 μm. **$P<0.01$, ***$P<0.001$ versus CON; ##$P<0.05$ versus DGA.
Fig. 8. Effects of paeonol on cofilin1 and p-cofilin1-immunoreactivity and their levels in frontal cortex. (A) Western blotting for cofilin1 and p-cofilin1 on Ser 3 in CON, DGA, and PAE. Paeonol downregulated the p-cofilin1 expression (C) and p-cofilin1/cofilin1 ratio (D), but did not affect the cofilin1 levels (B). Immunofluorescence labels of cofilin1 (red, excitation wavelength 543/emission wavelength bp560-615), p-cofilin1 (green excitation wave length 488/emission wave length bp500-530) and nucleus (blue, excitation wave length 458/emission wave length bp400-461) in frontal cortex (CON: E, F, K, L, and M; DGA: G, H, N, O, and P; PEA: I, J, Q, R, and S). The cells in (L), (O), and (R) are from (K), (N), and (Q), respectively. The fluorescence density curves of the single cell in (M), (P), and (S) were shown by the lines in (L), (O), and (R), respectively. White arrow in (Na) indicates the cofilin1-labeled dendrite similar to the “rod”. Data expressed as the means ± SEM (n=3). Scale bars in (Id) and (Qc) represent 10 μm and in (J) represent 5 μm. *P<0.05, **P<0.01 versus CON; *P<0.05 versus DGA.
Paeonol upregulated D-galactose and aluminum-induced decline of fibrillar actin in the frontal cortex

Actin filaments of ~200 nm diameter, which can be specifically labeled by phalloidin, constitute the dendritic spine cytoskeleton (Frost et al., 2010) and govern dendritic spine physiology by fibrillar actin (F-actin) nonequilibrium assembly and disassembly (Spence and Soderling, 2015). As shown in Fig. 7A, the rectangles represent the basal dendritic area and the apical dendrite area where the density of the actin filaments will be counted, respectively. One-way ANOVA indicated that both in the basal dendritic area ($F_{2,14}=20.324$, $P<0.001$) and the apical dendrite area ($F_{2,14}=20.907$, $P<0.001$), prominent differences in the density of actin filaments occurred between the groups. Treatment with paeonol significantly relieved D-galactose and aluminum-induced decline in the density of the actin filaments in the basal dendritic area (CON vs. DGA, $P<0.001$; PAE vs. CON, $P<0.01$; PAE vs. DGA, $P<0.01$) (Fig. 7D) as well as the apical dendrite area (CON vs. DGA, $P<0.001$; PAE vs. CON, $P<0.01$; PAE vs. DGA, $P<0.01$) (Fig. 7E).

Fig. 9. Effects of paeonol on the levels of RhoA, Rock2, and Limk1. (A) Western blot bands of the frontal cortex tissues determined with RhoA, Rock2, and Limk1 antibodies in CON (left column), DGA (middle column), and PAE (right column). GAPDH (36 KD) is an internal reference. Treatment with paeonol (50 mg/kg, i.p.) for 6 weeks significantly alleviated D-gal and AlCl3-induced upregulation of RhoA (B) and Rock2 (C), as well as tended to significantly reduce the expression levels of Limk1 (D). Data expressed as the means ± SEM (n=3). *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus CON; ###$P<0.001$ versus DGA.
Paeonol attenuated D-galactose and aluminum-induced alterations of cofilin1 in activity and redistribution in the frontal cortex

Cofilin1 as one of the best known regulators of actin remodeling (Pollard and Borisy, 2003; Bernstein and Bamburg, 2010), can be activated by dephosphorylation on Ser3 (Yang et al., 1998; Jose Javier et al., 2013) or release from membrane proteins (Shankar et al., 2007). Local excess inactivation or activation of cofilin1 may generate dendritic spine loss and dendrite atrophy (Van Troys et al., 2008; Bernstein and Bamburg, 2010). Therefore, in this study, we assessed the effects of paeonol on cofilin1 expression levels, activity, and redistribution in the frontal cortex.

Western blotting showed that D-galactose and aluminum did not change the cofilin1 expression levels ($F_{2,11} = 1.510, P > 0.05$), but did significantly increase p-cofilin1 levels ($F_{2,11} = 11.621, P < 0.01$) and p-cofilin1/cofilin1 ratio ($F_{2,11} = 14.639, P < 0.01$), these increases of which can be attenuated by paeonol treatment (p-cofilin1 and p-cofilin1/cofilin1 ratio: CON vs. DGA, $P < 0.01$; PAE vs. DGA, $P < 0.05$) (Fig. 8C and 8D).

Immunofluorescence revealed that cofilin1 immunoreactivity mainly distributed in the inner peri-membrane and neuronal processes, and p-cofilin1 was primarily found in the neuronal processes, in CON (Fig. 8E and 8F). In DGA, the distribution of cofilin1 translocated from the peri-membrane into the cytoplasm and nucleus, and p-cofilin1 prevalently distributed in the nuclei and neuronal processes (Fig. 8G and 8H). Paeonol evidently relieved D-galactose and aluminum-induced increased distribution of p-cofilin1 in the nucleus and neuronal processes (Fig. 8I-J), and of cofilin1 in the cytoplasm and nucleus (Fig. 8K-S). Some cells with high immunopositive cofilin1 or p-cofilin1 in the nucleus and some cells colocalized with cofilin1 and p-cofilin1 in the nucleus (Fig. 8G) were mainly observed in DGA, and were occasionally found in CON or PAE.

**Paeonol relieved D-galactose and aluminum-induced changes in RhoA, Rock2 and Limk1 levels in the frontal cortex**

Activation of Rho/Rock2/Limk1 pathway promotes the phosphorylation of cofilin1 (Bravo-Cordero et al., 2013). So, we examined the effects of paeonol on the expression of these signal molecules by western blotting. The significant differences between groups were found in these signal molecules, including RhoA ($F_{2,9} = 260.230, P < 0.001$), Rock2 ($F_{2,9} = 113.402, P < 0.001$), and Limk1 ($F_{2,9} = 6.301, P < 0.05$). Post hoc comparisons displayed that D-galactose and aluminum caused a significant upregulation in the expression levels of RhoA (CON vs. DGA, $P < 0.001$) (Fig. 9B), Rock2 (CON vs. DGA, $P < 0.001$) (Fig. 9C), and Limk1 (CON vs. DGA, $P < 0.05$) (Fig. 9D), these of which can be attenuated by treatment with paeonol (RhoA: PAE vs. DGA, $P < 0.001$; Rock2: PAE vs. DGA, $P < 0.01$; Limk1: PAE vs. DGA, $P = 0.081$) (Fig. 9B-D).

**DISCUSSION**

In the present study, we found that paeonol attenuated D-galactose and aluminum-induced behavioral dysfunction and AD-like pathological alterations in the frontal cortex. Accompanied by these changes were the alterations in the dendrite and dendritic spine densities as well as actin filaments. In addition, the activity and intracellular distribution of cofilin1 and the molecules of the signaling pathway that regulate cofilin1 phosphorylation have also changed. Our data suggests that paeonol may be through reducing Aβ levels to alleviate the loss of fibrillar actin and dendrites and dendritic spines via the Rho/Rock2/Limk1/cofilin1 signaling pathway in the frontal cortex, and ultimately improving AD-like behavior.

AD is characterized as either familial early-onset (EOAD; onset<65 years) or sporadic late-onset (sAD; onset > 65 years) (Drummond and Wisniewski, 2017). sAD afflicts > 95% of patients with AD (Bertram and Tanzi, 2012; Dong et al., 2014; Guerreiro and Hardy, 2014; Karch et al., 2014). It has been proposed that the combined effect of multiple factors is the most likely cause of AD formation, especially for sAD (Farrer et al., 1997; Qiu et al., 2004; Seripa et al., 2009; Wyss-Coray and Rogers, 2012; Orsucci et al., 2013; Solomon et al., 2014). In AD-related research, it is essential to develop appropriate animal models for validly evaluating the pathological processes, which are usually difficult to perform in patients (Van Dam and De Deyn, 2011). Currently, there are multiple categories of animal models used for AD-related research, and each animal model on AD-related research has its own advantages and limitations (Van Dam and De Deyn, 2011). However, the presence of both pathological features, Aβ and hyperphosphorylated-tau, is important to replicate the toxicity that occurs in human AD (Prins et al., 2010). Crosstalk between Aβ and tau can significantly increase their toxicity (Drummond and Wisniewski, 2017), leading to a decline in memory and visuo-spatial skills, as well as emotional dysfunction such as depression and anxiety (Lyketsos et al., 2011; Ameen-Ali et al., 2017; Götz et al., 2018). These are closely related with the damage in certain brain regions including the frontal cortex (Lyketsos et al., 2011; Götz et al., 2018). In this study, D-galactose and aluminum-induced animal model produced the
classical AD-like pathological features and behavioral performances, these of which can be significantly attenuated by treatment with paeonol.

A large amount of evidence has shown that synaptic loss is the best pathologic correlate of cognitive dysfunction in AD (Forner et al., 2017). Dendritic spines that protrude from the main dendritic shaft form more than 90% of excitatory synapses (Leuner and Gould, 2010), and synapse strength and activity are inseparably linked to spine morphology (Yasunori and Majewska, 2005). In the early 1990s, synapse and dendritic spine loss has been already observed by electron microscopy and densitometry of immunostained synaptic proteins in several brain areas of AD, including the prefrontal cortex, temporal cortex, and dentate gyrus of the hippocampus (DeKosky and Scheff, 1990; DeKosky et al., 1996; Terry et al., 2010). Since then, a series of similar results have been reported. In aged Tg2576 mice cortical neurons, spine elimination increases, and spine stability are markedly impaired leading to loss of synaptic structural integrity (Spires-Jones et al., 2007). In apolipoprotein E (APOE, the strongest genetic risk factor for sporadic AD) targeted replacement (TR) mice, the APOE4 TR mice have significantly reduced spine density and shortened spines in the cortex neurons compared with APOE3 TR mice and APOE2 TR mice (Dumanis et al., 2009). In transgenic mice expressing human tau (htau), a shift in spine morphology with fewer mushroom and more thin spines in both apical and basal dendrites of layer III pyramidal neurons from the prefrontal cortex occurs as a function of htau accumulation, and there is an overall decrease in volume of spines from 3 to 12 months (Dickstein et al., 2010). In 5xFAD mice, a significant loss of spines on basal dendrites is found in the somatosensory and prefrontal cortices, but not in the hippocampus (Crowe and Ellis-Davies, 2014). In human, spine density within layer II and III pyramidal neuron dendrites in Brodmann area 46 dorsolateral prefrontal cortex is similar among control and CAD cases (cognitively normal individuals with high AD pathology) but is reduced significantly in AD; thin and mushroom spines are reduced significantly in AD compared to CAD brains (Boros et al., 2017). Even at the end of AD, quantitative analysis indicates that more close correlation of postmortem cytopathology with premortem cognitive deficits is synapse and dendritic spine loss, rather than the numbers of plaques or tangles, degree of neuronal perikaryal loss, or extent of cortical gliosis (Selkoe, 2002; Wu et al., 2010). Similarly, our results showed that in frontal cortex, paeonol significantly relieved D-galactose and aluminum-induced reduction in the dendritic length and branches and dendritic spine density, especially the mushroom- and filopodia-type spines, suggesting that attenuation of spine loss by paeonol is mainly through reducing the loss of functional mature dendritic spines (mushroom) and increasing the production of new dendritic spines (filopodia).

It is well demonstrated that dendritic spine remodeling depends on the remodeling of cytoskeleton protein actin, which provides integrity for dendritic spines and organizes signaling machinery (Dillon and Goda, 2005; Schubert and Dotti, 2007; Bernstein and Bamberg, 2010; Sala and Segal, 2014; Maiti et al., 2015). Continuous actin polymerization into F-actin and depolymerization into globular actin (G-actin) are of major importance for the dynamic behavior of dendritic spines, contributing to their support, remodeling, and synaptic plasticity (Zhang and Benson, 2001; Star et al., 2002; Okamoto et al., 2004; Honkura et al., 2008). Therefore, it is well logical for our findings that the consistent alterations with dendrites and dendritic spines in the frontal cortex are F-actin specifically labeled by phalloidin.

In addition, actin also translocate into the nucleus (de Lanerolle and Serebryannyy, 2011; Dopie et al., 2012), in which nuclear actin is important in regulating gene accessibility, transcription, and post-transcriptional regulation (de Lanerolle and Serebryannyy, 2011). Studies have revealed that actin filament non-equilibrium assembly and disassembly is regulated by many factors including the actin-depolymerizing factor (ADF)/cofilin family that consists of three highly similar paralogs: ADF, cofilin1 (non-muscle cofilin), cofilin2 (muscle cofilin) (Bamburg and Wiggan, 2002; Bernstein and Bamberg, 2010). ADF/cofilin family has multiple functions and is called a functional node in cell biology (Bernstein and Bamberg, 2010). Cofilin1 is the most widely expressed member of the ADF/cofilin family, especially in mammal neurons (Bernstein and Bamberg, 2010), and is also considered as the most conserved and essential protein accelerating actin filament disassembly in cells (Vepsäläinen et al., 2013). Inhibition of cofilin1 is consequently lethal at the embryonic stage (Andrianantoandro and Pollard, 2006; Bernstein and Bamberg, 2010). Cofilin1 can be activated in a variety of ways, including dephosphorylation on its Ser3 and release of cofilin1 from membrane protein binding (Bernstein and Bamberg, 2010). However, misregulation of ADF/cofilin activity is linked to several pathologies (Yamaguchi and Condeelis, 2007; Wioland et al., 2017). Local excess activation or inactivation of cofilin may lead to dendritic spine loss and dendrite atrophy (Van Troys et al., 2008; Bernstein and Bamberg, 2010). In this study, we found that D-galactose and aluminum significantly elevated the p-cofilin1 levels, increased the distribution of cofilin1 translocating from the peri-membrane into the cytoplasm and nucleus, and/or aggrandized the p-cofilin1 prevalently distrib-
uting in the nuclei and neuronal processes (Fig. 8 G and H), these of which can be attenuated by treatment with paeonol. Our results suggest that the prevention of dendrite and dendritic spine loss by paeonol may be through regulating the activity of cofilin1 by affecting its local distribution in cells, and/or regulating the transcription of related genes in the nucleus via cofilin1 phosphorylation.

Early evidence has indicated that increased soluble Aβ oligomers (also known as Aβ-derived diffusible ligands, ADDLs), especially the Aβ oligomers at 56 kDa and pI 5.6, can induce synaptic loss in neurons in vitro and in vivo (Yuesong et al., 2003; Lacor et al., 2007; Ittner and Gotz, 2011), and highly correlate with impaired behaviors in AD (Sylvain et al., 2006). In vitro, soluble Aβ disrupts actin and microtubule dynamics via activation of RhoA and inhibition of histone deacetylase 6 (HDAC6) in cultured neurons (Tsushima et al., 2015). Aβ activates the RhoA GTPase by binding to p75NTR, and the inactivation of RhoA GTPase can protect cultured neurons against the noxious effects of Aβ (Chacon et al., 2011). In APP Tg mice, RhoA expression levels were increased in dystrophic neurites (Gema et al., 2009). Knockdown or pharmacologic inhibition of ROCK2, the downstream kinase of RhoA, decreased Aβ levels in the 5XFAD AD mouse (Hershkowitz et al., 2013). LIMK1 (LIM motif-containing protein kinase 1 is a serine/threonine kinase protein, also the downstream kinase of ROCK2, and can phosphorylate cofilin1 at Ser 3 forming inactivated p-cofilin1 (Scott et al., 2010; Bravo-Cordero et al., 2011) to regulate the neuronal morphology (Bernard, 2007). LIMK1 activation may play a key role in AD pathology (Heredia et al., 2006). The limk1 gene knockout mouse exhibits significant abnormalities in the morphology of dendritic spines and neurite growth cones and synaptic functions (Meng et al., 2002). Pharmacologic inhibition of LIMK1 provides dendritic spine resilience against β-amyloid (Henderson et al., 2019). These data indicate a RhoA-ROCK2-LIMK1-cofilin1 pathway really existed in the progress of AD. Our results that paeonol relieved D-galactose and aluminum-induced elevation of RhoA, ROCK2, and LIMK1 levels suggest this pathway involved in the protective effects of paeonol on dendrites and dendritic spines.

Additionally, studies reveal that LIMKs are inactivated by SSH1-mediated dephosphorylation (Soosaira-jah et al., 2014), and specific inhibitors for PI3K inhibit SSH1L activation (Nishita et al., 2004), indicating a negative relationship in the activity between LIMK1 and PI3K and/or SSH1. So, we examined the effects of paeonol on PI3K and SSH1 levels. Our results showed that paeonol remarkably relieved D-galactose and aluminum-induced downregulation of PI3K (Fig. 10B) and SSH1 (Fig. 10C) levels in the frontal cortex.

In conclusion, we have identified that paeonol has a protective role against dendrite atrophy and dendritic spine loss in frontal cortex. Furthermore, the protective efficacy is, at least in part, through the RhoA-ROCK2-LIMK1 pathway to regulate the cofilin1 activity and reduce the dendrite atrophy and dendritic spine loss, eventually attenuate the AD-like behavioral abnormalities.

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