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# Emodin attenuates Alzheimer's disease by activating the protein kinase C signaling pathway

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**Abstract:** To investigate the effects of emodin on learning and memory and protein kinase C (PKC) signaling pathway in Alzheimer's disease (AD) model mice. 60 APP/PS1 double transgenic AD mice were selected as model mice at the age of 7-8 months, 36 healthy male C57BL/6 mice served as the control group. Morris water maze method and passive avoidance experiment were used to evaluate the memory ability of mice. The thiazole blue (MTT) method and the lactate dehydrogenase (LDH) cytotoxicity test kit were used to evaluate the effect of emodin on the cell viability of hippocampal neurons in HT22 mice treated with β-amyloid peptide 1-42 (Aβ1-42). The effect of emodin on PKC levels was explored using the modified Takai method and Western blotting. Behavioral test results showed that the escape latency of the mice in the model group was longer than that in the control group ($P<0.05$), and the escape latency was significantly shortened given a emodin prognosis. The MTT and LDH test results showed that emodin to Aβ- overexpression induced the protective effect of hippocampus cells in HT22 mice. Western blot analysis showed that the phosphorylation level of PKC in mice increased significantly after emodin administration. Emodin can attenuate oxidative stress and inflammatory response in Alzheimer's model mice by activating PKC pathway, thereby improving cognitive function.

***Key words:*** Emodin; Alzheimer's disease; Protein kinase C; Learning and memory ability; HT22; Western blot.

## Introduction

Dementia is a persistent intellectual disorder with the complicated cause including genetic factors and acquired environmental influences. It can cause more than 100 kinds of diseases with dementia (1). Among them, there are two main types of dementia commonly suffered by the elderly: one is Alzheimer's disease (Alzheimer's disease, AD), the second is vascular dementia (2, 3). These two types account for more than 80% of senile dementia. Alzheimer's disease, first discovered by German doctors in 1906, hence the name (4). AD is a chronic central nervous system degenerative disease (5). The disease is characterized by progressive mental retardation, and its special pathological changes are senile plaques and neurofibrillary tangles in multiple brain regions, mainly hippocampus and cortical temporal lobe. The main component of senile plaques is amyloid beta, and neurofibrillary tangles are associated with hyperphosphorylation of Tau protein (6, 7). It is now believed that the etiology of AD is diverse, central neurotransmitter metabolism abnormalities, energy metabolism disorders, and amyloid metabolism abnormalities (8). Calcium imbalance in neurons, neuronal apoptosis, free radical damage, etc. play a role in the pathogenesis of AD (9-13). The complexity of AD etiology has caused the complexity of AD treatment, and there is currently no effective method for treating AD. A lot of research now shows that estrogen has the function of maintaining some advanced functions of the brain (14). It can improve the memory function in normal healthy women or AD patients. Estrogen in postmenopausal women can delay or reduce the occurrence of AD (15, 16). According to the theory that estrogen deficiency is one of the important reasons for AD, studies in the late 1980s proved that estrogen replacement therapy in postmenopausal women can effectively reduce the incidence of AD. However, considering the side effects of long-term use of estrogen, we are now looking for a way to increase both brain function and hormonal side effects.

Emodin is the most widely distributed monoterpenoid nucleus, 1, 6, 8-trihydroxyindole derivative, which is the main component of many traditional Chinese medicines. It is mainly derived from the dry roots and rhizomes of the palm leaf rhubarb. Studies have shown that it can play a role in anti-tumor, anti-inflammatory, diarrhea, scavenging oxygen free radicals, lipid-lowering, immune regulation, hemostasis, liver protection,

anti-renal fibrosis and other fields (17-22). However, emodin has a molecular structure similar to that of estrogen, and emodin has a strong free-trapping effect in cardiomyocytes and can maintain the antioxidant action of glutathione during oxidative stress; It is also possible to inhibit lipid peroxidation of the mitochondrial membrane. From this we conclude that emodin can have a protective effect on nerve cells similar to estrogen. It is now believed that amyloid precursor protein (APP) metabolic abnormality is the central link in the pathogenesis of AD. The protein kinase C (PKC) pathway is located upstream of the mitogen-activated protein kinase pathway and has neuroprotective effects by regulating AD-related amyloid degradation, it is considered to be an important signal transduction molecule that regulates APP metabolism, and is likely to be closely related to emodin-relieving AD function (23, 24). However, it is difficult to understand the neurogenicity of AD patients during disease progression for that brain tissue specimens from AD patients and normal controls are quite difficult to obtain. This study is to understand the neurological development as the AD progresses to help treat AD. The AD transgenic mouse model carrying APP and PS1 mutant genes showed major pathological changes in AD and memory loss. This model is very useful for the study of AD and the possible treatment of AD (25, 26). In this study, we used the APP/PS1 double-transgenic AD mouse model to study neurogenesis during AD progression. The effects of emodin on learning and memory ability and PKC signaling pathway in AD model mice were discussed.

## Materials and Methods

### Experimental animals and grouping

60 male healthy APP/PS1 transgenic mice aged 7-8 months were provided by the Nanjing Institute of Biomedical Research as an experimental group with a body weight of 28-30 g before the experiment. Thirty-six healthy male C57BL/6 mice were purchased from Shanghai Yisen Biotechnology Co., Ltd. as a control group. All experimental animals were kept at room temperature (23 ± 2) °C and humidity of 43% to 45% to ensure free access to drinking water. The treatment of the mice and all animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals recommended by the national institutes of health. Mice were randomly divided into 8 groups (12 mice in each group):

Group 1: C57BL/6 male mice without any treatment were evaluated for their behavioral parameters from day 64.

Group 2: C57BL/6 mice were intraperitoneally injected with dimethyl sulfoxide (0.5%, 10 mL/kg) for 1-70 days.

Group 3: C57BL/6 mice were intraperitoneally injected with emodin (20 mg/kg) dissolved in dimethyl sulfoxide (0.5%, 10 mL/kg) for 1-70 days.

Group 4: APP/PS1 transgenic mice without any treatment for 1-70 days.

Group 5, 6 and 7: APP/PS1 mice were intraperitoneally injected with three different doses of emodin (5, 10, 20 mg/kg) at 1-70 days respectively, and behavioral tests were conducted from day 64.

Group 8: APP/PS1 mice were intraperitoneally injected with emodin (20 mg/kg) at 1-70 days, but were supplemented with Calphostin C (selective PKC inhibitor) 1 hour before emodin treatment at 64-70 days.

### Reagents and cell lines

Aβ1-42 was purchased from Sigma Biotech Co., Ltd.; methylthiazolyldiphenyl-tetrazolium bromide (MTT) was purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd.; LDH cytotoxicity test kit was purchased from Shanghai Biyuntian Biotechnology Co., Ltd.; PKC The primary antibody (goat anti-mouse) was purchased from Shanghai Ruiqi Biotechnology Co., Ltd., and the second antibody was purchased from Beijing Kangwei Century Biotechnology Co., Ltd. HT22 mouse hippocampal neurons were purchased from Shanghai Kanglang Biotechnology Co., Ltd.

### Main instruments

$CO_2$ thermostatic incubator: Series II water Jacket, Thermo USA technology co., Ltd. Ultra-clean workbench: 1300 Series A2, Thermo technologies, USA; Enzyme marker: bio-rad company, USA; Vertical electrophoresis system, wet transfer system and basic power supply: bio-rad, USA; Protein gel imaging system: Shanghai tianneng technology co., Ltd.

### Behavioral testing

#### *Morris Water Maze Experiment*

Morris water maze method was used to evaluate the memory ability of mice. The Morris water maze consists of a circular water tank (180 cm in diameter and 70 cm high) with a constant temperature of 22 ± 1°C. The water tank is divided into four equal quadrants, north, west, south and east. The colorless escape platform (10 cm in diameter) is 2 cm below the water of the East quadrant, the target quadrant. For each group of mice, adaptive training was carried out in advance for 3 days, and then the time required for the mice from entering the water to finding the underwater concealed platform and standing on it was recorded for 3 consecutive days by using the positioning navigation experiment. The incubation period was denoted by seconds (s).

#### *Passive avoidance experiment*

Passive avoidance test was used to evaluate the memory ability of mice. At the beginning of the experiment, the experimental parameters, including safety period, conditioned stimulus time, unconditioned stimulus time and intensity, shuttle times, etc were first set. At the stage of memory acquisition, the rats were placed in a side chamber, close to and facing the endwall, and began to train after 5 minutes of adaptation. According to the preset program, the video analysis system of the shuttle experiment first gives the high-frequency stable sound stimulation and the flash stimulation. After a certain period of time, if the experimental mice still stay in the same side, then an electrical stimulation will be given. After receiving the electric shock, they will escape from the round hole to the opposite side for a period of time, and then the opposite side will be given conditional and unconditional stimulation, and so on. Each mouse was trained 30-50 times in a row until it

learned. After 24 hours, the mice were tested. After the end of all training, the computer showed the trajectory of each shuttle and recorded the avoidance response of the mice as the main evaluation index of their learning and memory performance.

### *MTT and LDH experiments*

To assess the effect of emodin on the activity of HT22 cells treated with -amyloid peptide 1-42 (A1-42), the effect of different concentrations of emodin (1, 5, 10, 20 M) on the survival rate of HT22 cells was firstly determined by MTT assay. Lactate dehydrogenase (LDH) is a stable protein found in the cytoplasm of normal cells. Once the cell membrane is damaged, LDH is released outside the cell. LDH catalytic lactic acid form pyruvic acid salt, and INT (tetrazolium salts) reaction form purple crystal material, so the effect of emodin on cytotoxicity was evaluated using the LDH Cytotoxicity Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) to assess the effect of emodin on cell toxicity, namely by selecting different concentrations of emodin respectively with HT22 hippocampal cells in mice after incubation for 1 hour, together by enzyme standard to test cell lactate dehydrogenase release, the release of lactate dehydrogenase was detected by a microplate reader. And the absorbance was measured at 540 nm and 490 nm by microplate microscopy.

### *Determination of PKC activity in hippocampus*

PKC activity assay was performed according to the modified Takai method. The mice were decapitated, and the brain tissue was quickly dissected. The hippocampus tissue was removed and placed in a sterile cryotube and stored in a refrigerator at -80 °C. Proteins were extracted from protein lysate (0.1 M Tris buffer, 10 mM EDTA, 10 mM DTT, Aprotinin 500 μg/mL, Leupeptin 500 μg/mL, Pepstatin A 500 μg/mL, 10 mM PMSF) for PKC activity determination. The cell membrane and plasma protein samples were taken at 10 g each, and the protein kinase assay buffer 25 μL was applied for 5 minutes. After that, it was spotted on Whatman filter, washed 3 times with 1 mL of phosphoric acid 10 mL, dried at 80 ° C, and 5 mL of PPO scintillation solution, and the cpm value was measured. The PKC activity was expressed by the sample CPM value-blank CPM value.

### *Western blotting experiments*

To detect the expression of PKC: 1) Remove the hippocampus from the ice, centrifuge the homogenate at the speed of 12000g, 4℃ for 2min, extract the total protein from the hippocampus, centrifuge the ultrasonic lysis, and separate the biological protein from the lysate; 2) Denature the protein component after homogenization, and the protein sample was electrophoresed by polyacrylamide gel (covering gel concentration 6%, separation gel concentration 10%) for 2 hours, and the separated protein was transferred to a polyvinylidene fluoride film (PVDF) (wet transfer 300 mA, 1.5 hours or 110 V, 1 hour); 3) After membrane transfer, incubate the membrane in 3% fetal bovine serum solution; 4) add primary antibody (1:200), incubate (4°C, overnight), wash the primary antibody in Tween-20 Tris buffer (TBST); 5) Incubate with secondary antibody (1:1000) (room temperature, 40 minutes), wash the secondary antibody in TBST, and illuminate. Image analysis was performed using NIH ImageJ software, and image grayscale was analyzed using the software Imagine.

### Statistical analysis

The experimental data processing results are expressed as mean standard deviation. Data analysis was performed using SPSS 22. 0 software for analysis of variance and multiple comparisons. The Student's test was used to determine the statistical significance of the difference, and a P value of less than 0.05 was significant.

## Results

### Effects of emodin on cognitive function of APP/PS1 mice

### *Morris Water Maze Experiment*

The results showed that the escape latency of the control group on day 66 was significantly reduced compared with the 64th day, indicating that the spatial learning ability was normal. However, APP/PS1 mice had significantly higher escape latency on day 66 compared to control mice, indicating learning impairment. Administration of emodin per day significantly and dose-dependently reduced the escape latency of APP/PS1 mice on day 66, which was significantly abolished by co-treatment with Calphostin C (Figure 1A). Furthermore, on day 67 of the study, mice in the control group spent significantly longer time in the target quadrant than other quadrants, indicating normal memory. APP/PS1 mice showed a significant reduction in the time spent in the target quadrant on day 67 and showed less crossover in the platform region compared to control mice, indicating memory impairment. Daily administration of emodin significantly and dose-dependently increased the time spent in the target quadrant on day 67 and the number of crossings in the APP/PS1 mouse platform region, which was significantly abolished by treatment with Calphostin C (Figure 1A- C).

### *Passive avoidance test*

All mice showed a normal mean initial latency on

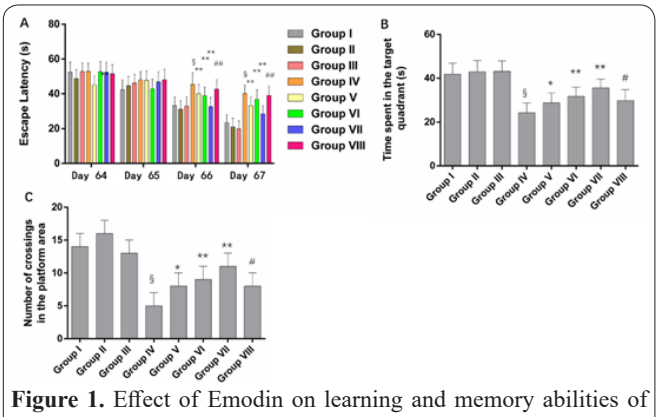


**Figure 1.** Effect of Emodin on learning and memory abilities of APP/PS1 mice assessed by the Morris water maze. A) Escape latency time during an acquisition trial; B) Mean time spent in the target quadrant on day 68 during a retrieval trial; C) Number of platform crossing n the target quadrant on day 68 during a retrieval trial. (n=12, **P*<0.05, ***P*<0.01 compared with group IV; $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared with group VII; $^{\S}P$<0.05, $^{\S\S}P$<0.01 compared with group I, II, III).

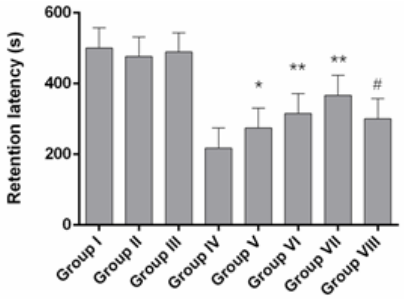


**Figure 2.** Effect of Emodin on the cognitive function of APP/PS1 mice assessed by the passive avoidance test on the day 70. n=12, **P*<0.05, ***P*<0.01 compared with group IV; #*P*<0.05 compared with group VII.

day 32, however, a significant change in the retention latency was observed on day 33 in the passive avoidance test. APP/PS1 mice had a significant reduction in retention latency on day 70 of the study compared to control mice, indicating impaired cognitive function. Daily emodin treatment significantly and dose-dependently increased the retention latency of APP/PS1 mice, which was significantly abolished by co-treatment with Calphostin C (Figure 2).

### *Emodin has a protective effect on the toxicity of Aβ-overexpression in hippocampus of HT22 mice*

Aβ is a major component of aging plaques and is formed in the brains of patients with Alzheimer's disease. It is also hypothesized that proteins formed by abnormal processing of this amyloid precursor protein are the main cause of neuronal death and disease. Aβ-induced apoptosis in hippocampus of HT22 mice increased, and our results showed that this effect was significantly reduced after emodin treatment at 10μM (as shown in Figure 3C). First, in order to quantify the neurotoxic effects of $A\beta_{1\text{-}42}$, HT22 cells were treated with various concentrations (1, 5, 10, 20 and 50 mM) of $A\beta_{1\text{-}42}$ for 24 hours (Fig. 3B). Cell viability was reduced by 60% after 24 hours of treatment with 5 mM $A\beta_{1\text{-}42}$. Therefore, 5 mM $A\beta_{1\text{-}42}$ was used in all subsequent experiments. MTT test results showed that HT22 cells treated with different concentrations of emodin (1, 5, 10 and 20 mM) for 24 hours showed no significant cell death (Fig. 3A). Pretreatment of HT22 cells with 10 mM emodin for 1 hour significantly increased HT22 activity before 5 mM Aβ1-42 was administered to HT22 cells (Fig. 3C). According to the LDH results, 10 mM emodin significantly inhibited $A\beta_{1\text{-}42}$-induced cytotoxicity in HT22 cells (Fig. 3D).

### *Effect of emodin on hippocampal PKC activity*

PKC is a family of serine/threonine protein kinases composed of multiple isoenzymes. It is a calcium-phosphorus-dependent protein kinase that catalyzes the phosphorylation of serine or threonine residues on various protein substrates. As one of the central molecules of the signaling pathway, PKC is distributed in both the membrane and the cytosol, and its amount is related to its active state. Takihino et al found that PKC activity was significantly reduced in brain tissue of patients with Alzheimer's disease. Therefore, Therefore, we searched for the effect of emodin on the activity of PKC in hippocampus. Figure 4 shows that the hippocampal PKC activity did not change much after 2 hours of emodin

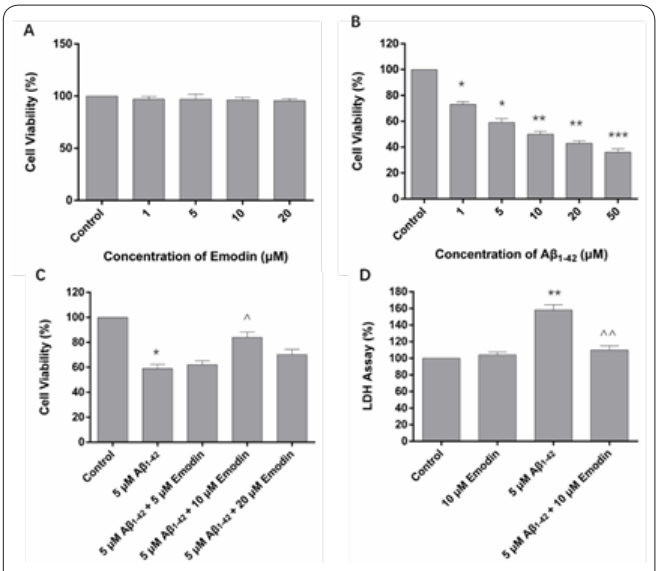


**Figure 3.** Protective effects of Emodin against Aβ-induced death of HT22 cells. (A, B) ViAβility of HT22 cells after treatment with increasing concentrations of Emodin (1-20 μM) or $A\beta_{1\text{-}42}$ (1-50 μM). (C) ViAβility of HT22 cells after treatment with 5 mM $A\beta_{1\text{-}42}$ for 24 h after pretreatment with Emodin (0, 5, 10, 20 μM) (D) Release of LDH into the culture supernatants after treatment with 5 μM $A\beta_{1\text{-}42}$ for 24 h after pretreatment with 10 μM Emodin. n=3, **P*<0.001, ***P*<0.01, ****P*<0.05, compared with control group; ^*P*<0.05, ^^*P*<0.01 compared with group 5 μM $A\beta_{1\text{-}42}$.

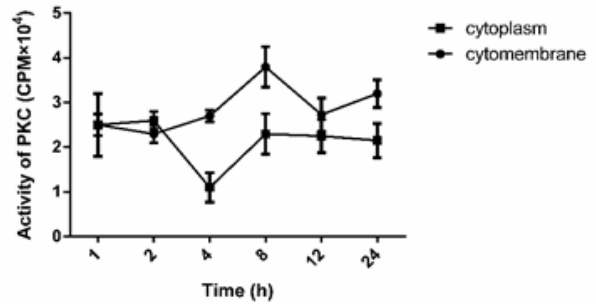


**Figure 4.** Effect of Emodin on PKC activity in hippocampus.

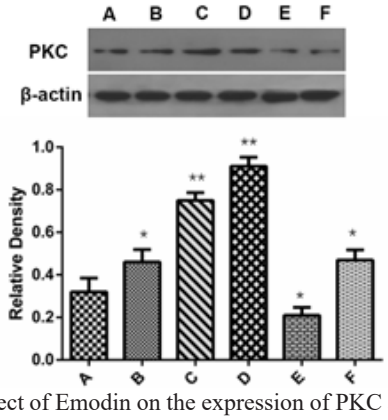


**Figure 5.** Effect of Emodin on the expression of PKC according to Western Blotting. Control group (A); Emodin (1 μM) group (B); Emodin (5 μM) group (C); Emodin (20 μM) group (D); Calphostin C (5 μM) group (E); and Emodin (20 μM) + Calphostin C (5 μM).β

administration. While after 4 hours, the cell membrane PKC activity increased significantly, and the peak value increased by about 4 hours, and the increase was about 52%.

### *Effect of emodin on the expression of protein kinase C*

As shown, pretreatment with emodin resulted in a significant increase in the protein level of PKC in cells

treated with $A\beta_{1-42}$, and an increase in the concentration of emodin and an increase in PKC expression (Figure 5). This result suggests that emodin can function by activating PKC. And compared with the single-administration PKC inhibitor Calphostin C group, the inhibition of PKC expression in the Calphostin C and emodin groups was also significantly alleviated. Therefore, our data indicate that emodin is an upstream activator of PKC in HT22 cells, and emodin can exert its neuroprotective effects by activating PKC.

## Discussion

AD is a neurodegenerative disease closely related to the elderly. The pathological mechanism is very complicated, and the pathogenesis is mostly related to aging and brain nerve cell reduction (27). More and more studies have found that the cause of AD patients is mostly Aβ deposition, senile plaque formation, which leads to brain neuron damage and death. Emodin is the most widely distributed monoterpenoid nucleus, 1,6,8-trihydroxyindole derivative, which has a similar molecular structure to estrogen, and emodin has a strong free capture effect in cardiomyocytes. And can maintain the antioxidant effect of glutathione during oxidative stress; it can also inhibit lipid peroxidation of mitochondrial membrane (22,23). From this we conclude that emodin can have a protective effect on nerve cells similar to estrogen. PKC is a family of serine/threonine protein kinases composed of multiple isoenzymes. It is a calcium-phosphorus-dependent protein kinase that catalyzes the phosphorylation of serine or threonine residues on various protein substrates. As one of the central molecules of the signaling pathway, PKC is distributed in both the membrane and the cytosol, and its amount is related to its active state. The study found that PKC activity was significantly reduced in brain tissue of AD patients (28). Therefore, this article is to explore the effect of emodin on AD through a series of experiments. From the results of the intelligence test, we learned that in the Morris water maze positioning experiment, the escape latency of APP/PS1 mice was significantly prolonged. In the passive avoidance experiment, the retention latency was significantly reduced, indicating the brain learning and memory ability of the model group. Seriously damaged, significantly reduced, suggesting that the model was selected successfully. Compared with the model group, the learning and memory ability of the emodin group was significantly improved, indicating that emodin can significantly improve the cognitive and memory impairment of dementia mice. PKC activity test results showed that the expression of PKC in hippocampus of AD rats was decreased, which was consistent with the decline of learning and memory scores. The emodin stimulation can significantly enhance the activity of PKC on the cell membrane of hippocampus. In addition, emodin significantly increased the protein level of PKC in the brain of APP/PS1 mice; a similar effect was observed in $A\beta_{1-42}$ treated HT22 cells. These novel results indicate that emodin can improve memory deficits in vivo and that this improvement is accompanied by a decrease in amyloid plaques. In summary, we demonstrate that emodin exerts neuroprotective effects by activating the hippocampal PKC pathway and slows AD.

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None.

**Conflict of interest**
There are no conflicts of interest in this study.

**Author's contribution**
All work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its content. The study was conceived and designed by Maode Wang; Changwang Du, Luoning Shi, Mei Wang, Ping Mao, Jia Wang, Yanqiang Wei, Juanru Hou and Maode Wang collected and analysed the data; Changwang Du wrote the text and all authors have read and approved the text prior to publication.

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