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Acetylpuerarin reduces inflammation and improves memory function in a rat model of Alzheimer's disease induced by $A\beta_{1\text{-}42}$

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This study was performed to determine if acetylpuerarin (compound N-2211) could reduce amyloid-$\beta_{1\text{-}42}$ ($A\beta_{1\text{-}42}$) induced learning and memory deficits and to examine its anti-neuroinflammatory effects in a rat model. Forty Wistar rats were randomly divided into four groups (n = 10 each): control, model ($A\beta_{1\text{-}42}$ injected), low-dose and high-dose acetylpuerarin groups. The acetylpuerarin groups received peritoneal acetylpuerarin every day for 12 days after 2 weeks of $A\beta_{1\text{-}42}$ (5 μg/1 μl) intrahippocampal injections. The Morris water maze (MWM) was used to assess rats' learning and memory abilities. Immunohistochemistry was used to assess expression levels of ionized calcium-binding adaptor molecule (Iba1), protein kinase C delta (PKCδ), IκB kinase β (IKKβ), and inducible nitric oxide synthase (iNOS) in hippocampus. After $A\beta_{1\text{-}42}$ injection, the learning and memory abilities of rats were reduced, and acetylpuerarin treatment ameliorated the observed deficits. $A\beta_{1\text{-}42}$ injection resulted in microglia transforming from resting microglia into an activated state, but this was reduced by acetylpuerarin treatment. Furthermore, hippocampal expression of PKCδ, IKKβ, and iNOS increased following $A\beta_{1\text{-}42}$ treatment, and acetylpuerarin could suppressed the levels of PKCδ, iNOS, and IKKβ. Acetylpuerarin improves learning and memory functions in $A\beta_{1\text{-}42}$ induced rat models. These effects may be due to anti-neuroinflammatory effects.

1. Introduction

Alzheimer's disease (AD) is one of the most serious neurodegenerative diseases affecting the aging population and leading to severe cognitive dysfunction. One hypothesis of AD pathogenesis is the neuroinflammation theory, which suggests that inflammatory processes trigger neurodegeneration signaling pathways and exacerbate amyloid-β (Aβ) plaque assembly (Burguillos et al. 2011; Block et al. 2007). It has been shown that accumulation of Aβ, especially the $A\beta_{1\text{-}42}$ peptide, in the brain initiates a cascade of events that ultimately leads to neuronal dysfunction, neurodegeneration, and dementia (Klafki et al. 2006; McLarnon and Ryu 2008).

Acetylpuerarin is a major isoflavonoid derived from the Chinese medical herb *Radix puerariae*, and its structure is desired from puerarin (Fig. 1). It is more lipid-soluble than puerarin and can successfully cross the blood-brain barrier (BBB). Our previous studies (Hou et al. 2007; Hou et al. 2004; Liu et al. 2007; Li et al. 2005) have demonstrated that acetylpuerarin has protective effects against brain ischemia-reperfusion injury. However, the potential effects of acetylpuerarin on AD-related pathogenesis and inflammation have not been investigated. This study was designed to examine whether acetylpuerarin is neuroprotective in an AD model and to determine if the effects are induced by suppressing neuroinflammation *via* activated microglia and the PKCδ/IKK/NF-κB pathway. We tested our hypothesis by performing intra-hippocampal injections of $A\beta_{1\text{-}42}$ in rats and determining if acetylpuerarin ameliorated $A\beta_{1\text{-}42}$ induced learning and memory impairments. We specifically assessed the roles of PKCδ, IKKβ, and iNOS.

2. Investigations and results

2.1. Acetylpuerarin improved learning and memory

We tested escape latency and target area exploring time in the Morris water maze (MWM) before surgery, 2 weeks after surgery, and 26 days after surgery (after treatment). We found that the $A\beta_{1\text{-}42}$ treated rats were significantly impaired 2 weeks after surgery; compared with the control group, their mean escape latency was significantly increased in the place navigation test (Fig. 2) ($P < 0.01$), and mean exploring time in the target area was significantly decreased in the spatial probe test (Fig. 4) ($P < 0.01$). After 12 days of acetylpuerarin treatment, we found that both doses significantly attenuated $A\beta_{1\text{-}42}$ induced learning and memory deficits, as evidenced by the decreased escape latency in the place navigation test (Fig. 2) and increased mean exploring time in the target area in the spatial probe test (Figs. 3-4).

We did not observe any changes in locomotion during the MWM, suggesting that the increased time was due to impaired learning rather than difficulty in swimming.

2.2. Acetylpuerarin reduced $A\beta_{1\text{-}42}$-induced microglial activation

Microglia were visualized with an antibody against the ionized calcium-binding adaptor molecule (Iba1), which is a microglia-

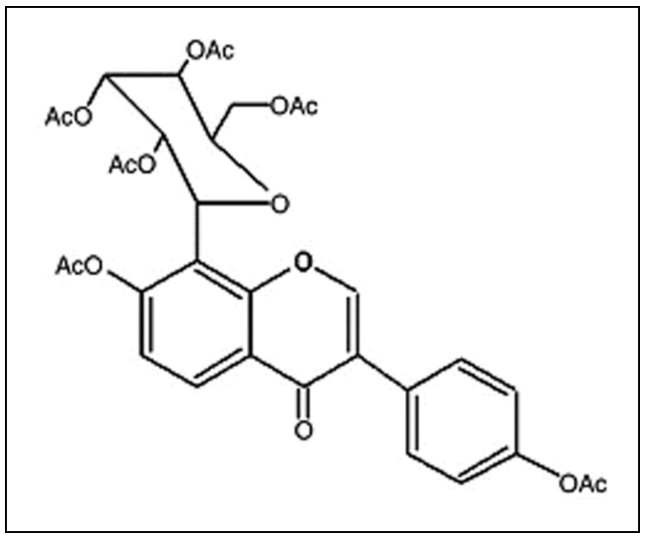

Fig. 1: Chemical structure of acetylpuerarin.

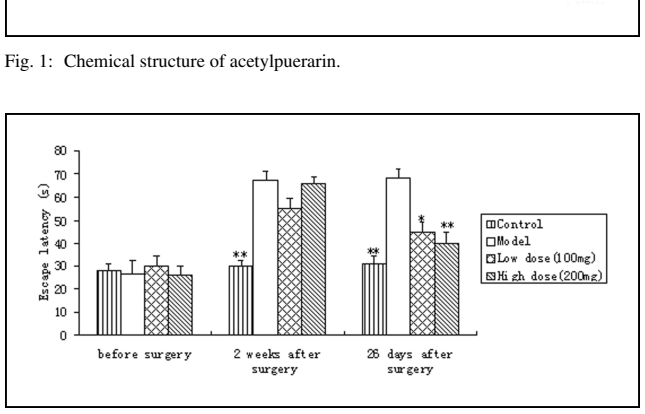

Fig. 2: Place navigation test. The escape latency time for different groups of rats to find the hidden platform in the MWM before surgery, 2 weeks after surgery, and 26 days after surgery (after treatment) (*$P < 0.05$, **$P < 0.01$, n = 10/group). Learning and memory abilities in both treated groups were significantly improved after 12 days of acetylpuerarin treatment.

specific marker. $A\beta_{1\text{-}42}$ treatment resulted in microglial activation in cerebral cortex. This process is characterized by morphological changes, such as short, thick dendritic protrusions and extended cell bodies that resemble macrophages.

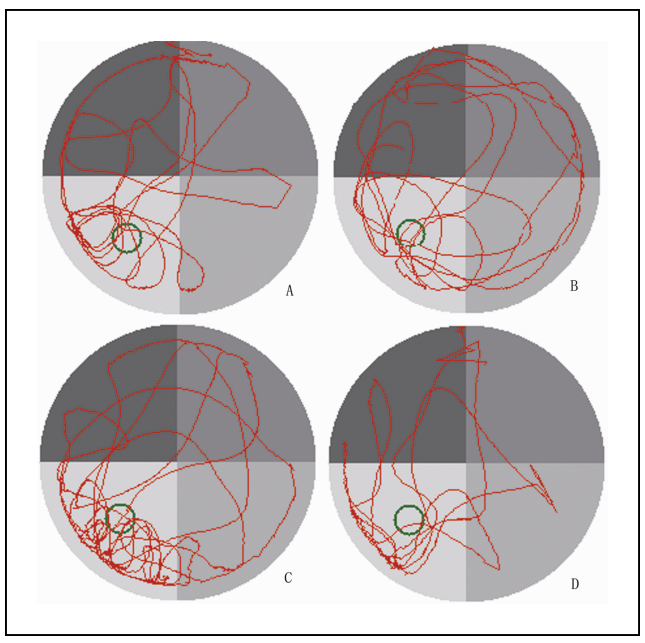

Fig. 3: Spatial probe test swim traces. Representative swim traces for each group in probe trials: (A) Control, (B) Model, (C) Low-dose acetylpuerarin, (D) High-dose acetylpuerarin.

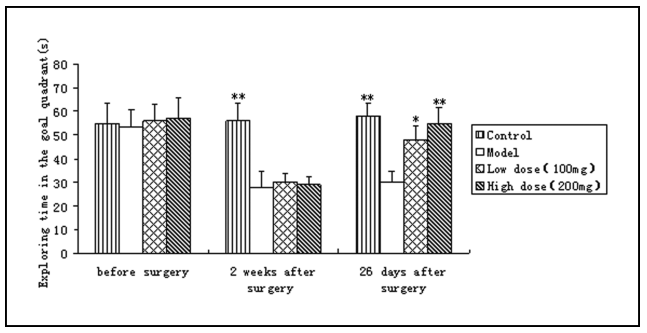

Fig. 4: Spatial probe test. Total exploring time in the goal quadrant of the MWM for different groups of rats before surgery, 2 weeks after surgery, and 26 days after surgery (after treatment) (*$P < 0.05$, **$P < 0.01$, n = 10/group). Rats in the acetylpuerarin-treated group spent more time in the target quadrant compared to the model group.

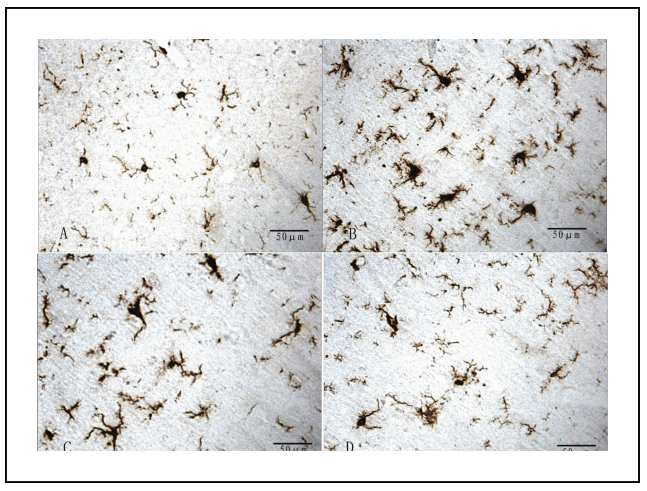

Fig. 5: Representative immunohistochemistry for Iba1. Control (A), model (B), low-dose acetylpuerarin (C), and high-dose acetylpuerarin (D), light microscopy, 400X. $A\beta_{1\text{-}42}$ stimulation resulted in microglial proliferation and activation in the cerebral cortex, characterized by morphological changes, such as short, thick dendritic protrusions and extended cell bodies that resemble macrophages. Acetylpuerarin treatment significantly inhibited $A\beta_{1\text{-}42}$ induced microglia changes in cerebral cortex.

Acetylpuerarin significantly inhibited $A\beta_{1\text{-}42}$ induced microglia changes in cerebral cortex (Fig. 5, Fig. 6).

2.3. Acetylpuerarin suppressed PKCδ, IKKβ, and iNOS expression

PKCδ, IKKβ, and iNOS were selected as neuroinflammation related markers in this study. In all brain tissue slices, distinct staining of the cytoplasm and membrane of CA1 pyramidal neurons was observed, indicating immunoreactiv-

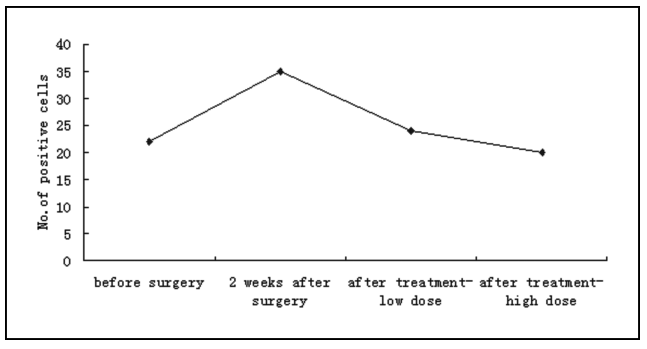

Fig. 6: Quantitative assessment of Iba1-positive cells.

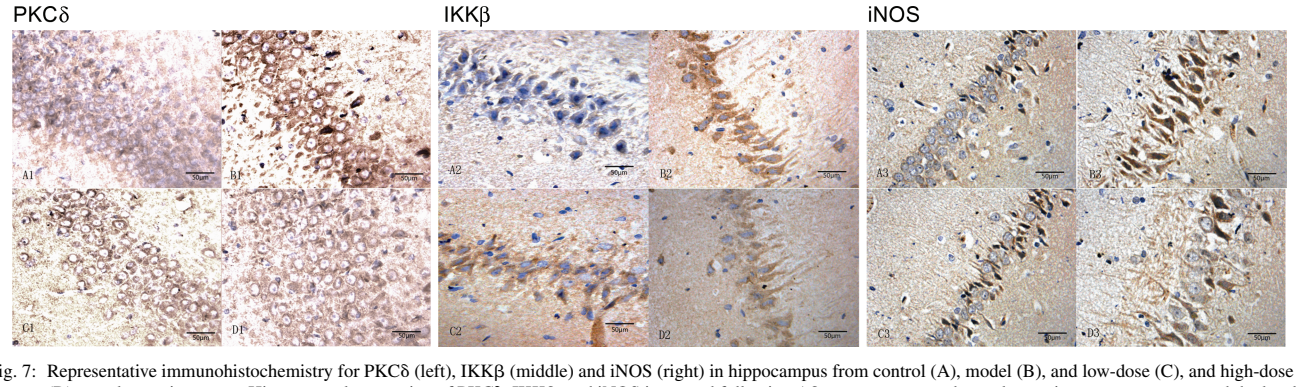

Fig. 7: Representative immunohistochemistry for PKCδ (left), IKKβ (middle) and iNOS (right) in hippocampus from control (A), model (B), and low-dose (C), and high-dose (D) acetylpuerarin groups. Hippocampal expression of PKCδ, IKKβ, and iNOS increased following $A\beta_{1\text{-}42}$ treatment, and acetylpuerarin treatment suppressed the levels of PKCδ, iNOS, and IKKβ.

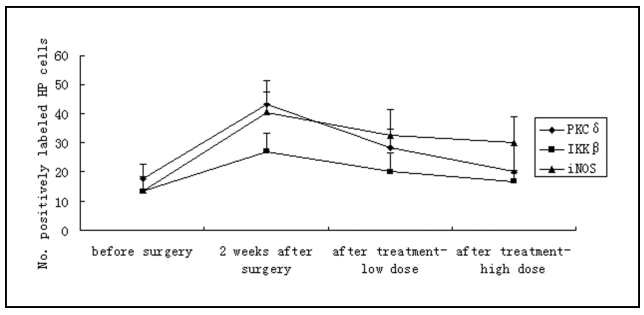

Fig. 8: Quantitative assessment of PKCδ-, IKKβ- and iNOS-positive hippocampal pyramidal cells.

ity for PKCδ, IKKβ, and iNOS (Fig. 7, 8). We assessed the number of PKCδ-positive hippocampal cells in the four groups, the values of which are as follows: model, 43.22 ± 8.14 ($P < 0.01$ compared to control); control, 17.76 ± 4.97, low-dose acetylpuerarin, 28.43 ± 6.34 ($P < 0.05$ compared to model); and high-dose acetylpuerarin, 20.17 ± 8.98 ($P < 0.01$ compared to model). Similar results were observed in IKKβ- and iNOS-stained sections. The numbers of IKKβ- positive hippocampal cells for the four groups were: model, 27.12 ± 6.15 ($P < 0.01$ compared to control); control, 13.39 ± 4.39, low-dose acetylpuerarin, 20.13 ± 6.32 ($P < 0.01$ compared to model); and high-dose acetylpuerarin, 16.78 ± 4.08 ($P < 0.01$ compared to model). The numbers of iNOS-positive cells were as follows: model, 39.21 ± 7.15 ($P < 0.01$ compared to control); control, 13.73 ± 4.74 ($P < 0.01$ compared to control); low-dose acetylpuerarin, 32.63 ± 8.78; and high-dose acetylpuerarin 29.98 ± 9.07 ($P < 0.01$ compared to model).

3. Discussion

$A\beta_{1\text{-}42}$ injection into hippocampal and cortical regions of brain have utility as an animal model of AD and have been used to study the inflammatory component of disease pathology (McLarnon and Ryu 2008). Cellular interactions with the peptide activate microglia and elicit chemotactic and proliferative microglia-mediated inflammatory responses. Numerous chemokine and cytokine factors likely initiate autocrine and paracrine cellular signaling processes that result in chronic inflammation. Proinflammatory cytokines and reactive oxygen species produced by activated microglia reduce neuronal viability. In this *in vivo* study, we found that bilateral hippocampal injection of $A\beta_{1\text{-}42}$ reduced learning and memory, stimulated microglial activation and proliferation, and significantly increased PKCδ, iNOS, and IKKβ expression in the rat hippocampus. This result is consistent with previous studies. Thus, the activation of counter regulatory mechanisms is essential to avoid the escalation of central nervous system inflammatory processes, anti-inflammatory strategies have continually and increasingly been considered to reduce the risks of various neuropathologies, including AD.

Microglial cells are the brain's resident macrophages and play important roles in immune and inflammatory responses. They signal other glial and neuronal cells *via* cytokines by secreting a variety of immune-related substances, generate free radicals, and are responsible for clearing amyloid deposits. Reactive microglial products mediate astrocyte activation and neuronal injury, and microglial inflammatory responses may have deleterious effects on neurons (Block et al. 2007; Burguillos et al. 2011). Iba1 is a microglia/macrophage-specific calcium-binding protein that has actin-bundling activity and participates in membrane ruffling and phagocytosis in activated microglia (Kanazawa et al. 2002). In our study, Iba1 immunoreactivity demonstrated that acetylpuerarin inhibited $A\beta_{1\text{-}42}$-induced microglia proliferation and activation.

IKB kinases (IKKα, IKKβ) play an important role in the activation of NF-κB, and the IKKβ/NF-κB pathway is the key signal transduction pathway for inflammatory responses. In the present study, we employed IKKβ as an inflammation marker and found that $A\beta_{1\text{-}42}$ induced IKKβ upregulation and acetylpuerarin down-regulates IKKβ, this may block the NF-κB signaling cascade. iNOS is a responsive enzyme in inflammation; its upregulation is not only a proliferation marker of responsive glial, it is also related to neuronal damage. iNOS can continuously catalyze NO production, which can cause neurotoxicity and lead to neuron necrosis or apoptosis (Haas et al. 2002; Steinert et al. 2010). Some studies (Burguillos et al. 2011; Wang et al. 2011) have demonstrated that reduced learning and memory in rats is related to neurotoxicity induced by NOS overexpression. Drugs that suppress nNOS and iNOS activity or expression can protect neurons and improve learning and memory in rats. In the present study, we found that that $A\beta_{1\text{-}42}$ induced iNOS up-regulation and acetylpuerarin ameliorated this increase.

PKCδ, a novel protein kinase C, has been reported to regulate NF-κB activation through IKK complexes and phosphorylation of the NF-κB inhibitor IkB (Ramnath et al. 2010; Storz et al. 2004; Vancurova et al. 2001). A recent study (Burguillos et al. 2011) demonstrated that PKCδ activation is important in microglia activation; activated caspase-3/7 interacts with the IKK/NF-κB pathway via PKCδ during LPS-induced microglia activation, and inhibition of PKCδ significantly decreased LPS-induced iNOS expression. Blocking PKCδ represents a promising prophylactic and/or therapeutic tool for treating neurodegenerative diseases. In the present work, we found that $A\beta_{1\text{-}42}$ induced PKCδ upregulation and this was ameliorated by acetylpuerarin. Our results suggest that PKCδ suppression may inhibit microglial activation and inflammation.

Acetylpuerarin is a new type of isoflavonoid compounds (N-2211) created by introducing a latent chain into puerarin. This modification increases liposolubility and allows it to cross the BBB. Previous studies showed acetylpuerarin's protective effects against brain ischemia-reperfusion injury depending on its antioxidant property, anti-apoptosis (Hou et al. 2007, 2004; Li et al. 2005; Liu et al. 2011). Few studies have assessed the impact of acetylpuerarin on inflammatory responses. Here, we showed that acetylpuerarin rescues MWM learning in rats, inhibits $A\beta_{1\text{-}42}$-induced microglia proliferation and activation, and reduces PKCδ, IKKβ and iNOS expression in $A\beta_{1\text{-}42}$-treated hippocampus tissue. By demonstrating the suppressive activities of acetylpuerarin on diverse inflammatory mediators through a PKCδ-dependent pathway, our results broaden the potential pharmacological utility of acetylpuerarin in treating inflammation related to neurodegeneration.

4. Experimental

4.1. Drugs and reagents

Acetylpuerarin was obtained from the Shandong Academy of Medical Science (MW: 668 D. Lot: 2002-12-2211). Lyophilized $A\beta_{1\text{-}42}$ (Sigma-Aldrich, Beijing, China) was prepared at a concentration of 5 μg/μl, and the aggregated form was prepared by placing the peptide solution in an incubator at 37 °C for 72 h. Acetylpuerarin was dissolved in distilled water at the appropriate concentrations. An antibody to PKCδ (ab47473) was purchased from Abcam Biochemicals, Cambridge, UK, and antibodys to IKKβ (BS1407), to iNOS(BS1821) were purchased from Bioworld Technology. Streptavidin-biotin complex and diaminobenzidine (DAB) staining kits were purchased from Zhongshan Jinqiao Biological Engineering Co. (Beijing, China). All other laboratory chemicals were of reagent grade.

4.2. Animals

Seven-month-old female Wistar rats (350-380 g) were supplied by the Animal Center of Lunan Biotechnology Ltd. (Jining, Shandong, China; SPF Grade II). The animals were kept in a regulated environment (23 ± 1 °C, 50 ± 2% humidity) with a 12-h light/dark cycle (lights on 8:00 a.m.-8:00 p.m.). All animal studies were performed in compliance with Animal Care and Use Guidelines in China.

4.3. Study design

Forty rats were randomly divided into four groups (n = 10 each): (1) control group; (2) model group ($A\beta_{1\text{-}42}$ intrahippocampal injection (3) low-dose acetylpuerarin group ($A\beta_{1\text{-}42}$ intrahippocampal injection plus 100 mg/kg acetylpuerarin); and (4) high-dose acetylpuerarin group ($A\beta_{1\text{-}42}$ intrahippocampal injection plus 200 mg/kg acetylpuerarin). For drug treatment group rats, acetylpuerarin (100, 200 mg/kg) was administered by intraperitoneal injection every day for 12 days. The control and model rats received intraperitoneal distilled water for the same amount of time.

4.4. Surgical procedures

Bilateral intrahippocampal Aβ injections were carried out as described previously (McLarnon and Ryu 2008) with minor modifications. Briefly, rats were anesthetized with 10% chloral hydrate (35 mg/kg, intraperitoneal injection) and placed in a stereotaxic apparatus. The scalp was incised and retracted, and a dental drill was used to place holes at appropriate locations in the skull. Coordinates for the hippocampal infusions were as follows: 3.5 mm posterior to lambda, 2.5 mm lateral to the mid-line, and 3 mm ventral to the meninges. $A\beta_{1\text{-}42}$ (5 μg in 1 μl sterile saline) infusions were performed at a rate of 1 μl/min with a 5-μl microinjector. The control group was injected with 2 μl sterile saline at a rate of 1 μl/min. For both treatments, the syringe remained in place for 5 min after the injection.

4.5. Morris water maze (MWM) test

After the 12-day drug treatment, the MWM was administered to learning and memory abilities as previously described (Wang et al. 2011). The MWM test included two parts. The first was the place navigation test from day 1 to day 5. On the first day, the rats were acclimated to the maze (without platform) for 2 min. On each of the next four days, rats were trained four times in the morning and four times in the afternoon with 4-min intervals. The platform was placed in the southwest quadrant area, and the starting point was changed for every training period. The routes and times taken to find the platform were recorded. The second portion of the MWM was the spatial probe test on day 6. The platform was removed after the spatial navigation test, and the rats' ability to find the removed platform by memory was tested. The time spent in the correct quadrant (i.e., where the platform was located during the training sessions) was recorded, and the percentage of the total time was calculated.

4.6. Brain sample preparation

Brain samples were collected after MWM testing. Rats were anesthetized with 35% chloral hydrate solution and fixed in a supine. The chest was opened to fully expose thorax and heart. A blunt infusion needle connected to a transfusion bottle was inserted into the aorta through the tip of the heart. The ascending aorta was clamped, and the right auricle of heart was cut open, and the animal was perfused with cold (4 °C) normal saline. When the outflow of normal saline from the right atria was clear, it was replaced by cold paraformaldehyde (4 °C) until the body became rigid. The brain was removed and fixed in paraformaldehyde (4 °C) over night. The next day, the tissues were removed and deposited in 20% sucrose solution containing antiseptics.

4.7. Immunohistochemistry

For immunohistochemistry, sections were rehydrated, microwaved in 10 mM citrate buffer (pH 6.0) for 15 min at 800 W for antigen retrieval, and incubated with anti-Iba1(1:250), anti-PKCδ (1:50), anti-IKKβ (1:100), or anti-iNOS (1:50) at 4 °C overnight before DAB visualization. For PKCδ, IKKβ, and iNOS expression, three sections of each hippocampi (six sections from each rat) were used for cell counting. The sections were viewed under a 40X objective, five random regions of the hippocampus CA1 were imaged, and these areas were used to count the immune-positive cells. For Iba1 expression, we counted the Iba1 positive cells in cerebral cortex. Each section was counted by three persons blindly.

4.8. Statistical processing

Values are given as mean ± standard deviation, and analysis of variance and t-tests were performed with SPSS13.0 software. $P < 0.05$ was considered to be statistically significant.

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