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Original Article

The effects of *Porphyromonas gingivalis* on the apoptosis of hippocampal cells in Sprague-Dawley rats and its underlying mechanisms

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Abstract: Neuron loss caused by neuronal apoptosis is one of the main pathological changes of Alzheimer's disease (AD). As a common infectious disease, chronic periodontitis has been found to be closely related to the occurrence and development of AD. *Porphyromonas gingivalis* (*P. gingivalis*) is one of the main pathogenic bacteria of periodontitis. In this study, we used a *P. gingivalis* peripheral infection model to study the effects and mechanisms of *P. gingivalis* on the apoptosis of hippocampal cells in Sprague-Dawley rats. After intravenous injections of *P. gingivalis* for 4 and 12 weeks, hematoxylin & eosin staining showed that the morphology of the cells in the hippocampus of the experimental group was changed. Nissl staining showed that cells in the hippocampus of the experimental group were loosely arranged and that the number of Nissl bodies was reduced. The TUNEL method revealed different levels of cell apoptosis in the experimental group and an increased expression of the cleaved caspase-3 protein, which indicates the existence of apoptosis. To explore the possible mechanisms of the cell apoptosis, we measured the expression levels of the N-methyl-D-aspartate receptor subunit NR2B and the postsynaptic density protein PSD-95, and the results showed increased expressions of both proteins. Elevated intracellular calcium concentrations were also detected in the hippocampal cells. In conclusion, we speculated that the high expression of the NR2B and PSD-95 proteins in the hippocampus caused by venous infection with *P. gingivalis* induced excitatory amino acid toxicity, leading to an extracellular calcium influx in the hippocampus, and eventually inducing apoptosis.

Keywords: *Porphyromonas gingivalis*, Alzheimer's disease, periodontitis, apoptosis, calcium ion, N-Methyl-D-aspartate receptor

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder and the most common type of dementia. The clinical manifestations of AD are progressive memory impairment, cognitive dysfunction, language impairment and other neuropsychiatric symptoms. Pathologically, AD is characterized by the formation of intracellular neurofibrillary tangles, the deposition of extracellular senile plaques within the afflicted brains and neuron loss [1]. The disease includes early onset AD and sporadic late onset AD (SLOAD). Early onset AD is associated with genetic mutations. SLOAD is the most common form of AD, accounting for approximately 98% of all cases [2]. The pathogenesis of SLOAD is complex and includes immune and environmental factors [3, 4]. Recent studies have shown that peripheral microbiological infections, such as *Chlamydia pneumoniae, Herpes zoster virus,* and *Treponema pallidum*, may be a potential cause of SLOAD.

Periodontitis is a chronic infectious disease with a large range of lesions, and it is the most common type of disease in humans worldwide [5]. In addition to the partial destruction of periodontal tissues, traditional oral hygiene procedures, tooth extraction and the injection of dental anesthetics can cause bacteria, bacterial toxicity products and inflammatory factors to be transplanted to other parts of the body through periodontal pockets. These translocated bacteria and toxins are associated with a variety of systemic diseases, including atherosclerosis, rheumatoid arthritis, diabetes, and AD [6]. In a 32-year-old cohort study, researchers found that lack of teeth, periodontal pockets and loss of alveolar bone were associated

with cognitive impairment, and this phenomenon is more obvious in subjects over the age of 45. Periodontal status and the number of missing teeth can predict the severity of cognitive impairment [7]. Another epidemiologic study investigated the association between oral health and cognitive function in young, middle-aged, and older adults in a cohort of 5,138 people aged between 20 and 59 years; after a full adjustment for all other covariates, gingival bleeding and loss of periodontal attachment were significantly associated with cognitive impairment [8]. Noble et al. found that serum IgG levels in response to common periodontal microbiota are associated with a risk for the development of incident AD [9]. Some researchers selected transgenic AD mice to establish a periodontitis model and observed that periodontitis can promote the expression of cytokine deposition and inflammatory factors in the hippocampal tissues of AD mice. However, as the transgenic mouse model only simulates early onset AD, the effect of periodontitis on late sporadic AD has not been indicated [10].

Neuronal apoptosis is associated with AD. The number of apoptotic neurons in the brain tissue of AD patients is increased, and the loss of neurons caused by apoptosis is one of the pathological features of AD [11]. Neuron loss is the main cause of the occurrence and aggravation of cognitive impairment [12], and it is associated with many factors. Damage to the mitochondrial function and structure leads to excessive oxidative stress, which can lead to neuronal apoptosis [13]. β-amyloid deposition and Tau protein hyperphosphorylation can activate glial cells and further promote neuronal apoptosis [14, 15]. The homeostasis of the calcium ion channels on the cell surface changes, resulting in a large amount of extracellular calcium ion influx, leading to the process of apoptosis induced by intracellular calcium overload [16]. It has been proposed that the calcium overload caused by the activation of the N-methyl-D-aspartic acid receptor is related to neuronal apoptosis.

The N-methyl-D-aspartate receptor (NMDAR) is an important excitatory amino acid receptor in the central nervous system; it is a ligand-gated ion channel that can bind magnesium ions to prevent the internal flow of calcium ions in the resting state [17]. Excitatory amino acids are widely found in the central nervous systems of mammals, including glutamate and aspartic acid, among which glutamate is the most abundant excitatory amino acid in the central nervous system. Studies have shown that glutamic acid and its receptor participate in the transmission of neuronal information, which is closely related to the formation mechanism of learning and memory as well as cognitive function [18, 19]. Excitatory neurotoxicity is the process of neuronal death resulting from the excessive and sustained activation of the receptor for the excitatory neurotransmitter glutamate [20]. NMDARs and its mediated calcium overload play key roles in glutamate excitatory neurotoxicity [21]. The neurotoxic effects of NMDAR-mediated calcium overload are associated with various neurodegenerative lesions, including AD. A large number of studies have shown that NR2B-NMDAR overexpression or overactivation can promote an extracellular calcium influx, which leads to intracellular calcium overload and glutamate excitatory toxicity, triggering the apoptosis of neuronal cells. The responses of neurons to glutamate or NMDA follow a bell-shaped curve, as both too much and too little NMDA activity are potentially harmful [20].

Postsynaptic density protein-95 (PSD-95) is the major scaffolding protein of postsynaptic glutamatergic synapses, and it contains various combinations of protein domains, including 3 PDZ domains, an SH3 domain, and the guanylate kinase domain [22, 23]. NMDAR binds to other signaling molecules, such as protein kinases, to form receptor protein complexes in the postsynaptic membrane via the postsynaptic scaffold protein PSD-95. It has been shown that the PSD-95 protein can interact with the upstream activator Pyk2 through the PDZ and SH3 domains, regulating Src PTK (Src family protein tyrosine kinases) activity and leading to the phosphorylation of the NR2 subunit, thus increasing the function of NMDAR [24-26]. In addition, PSD-95 forms complexes with CaMKII/GluR6 and GluR5/nNOS, activates JNK (c-Jun N-terminal kinases) signaling pathways, and is involved in neuronal apoptosis after brain injury [27, 28].

A number of studies support the idea that *P. gingivalis* is a master evader of the host's immune system [29]. *P. gingivalis* infection is associated with the onset of AD. It can enter the blood circulation system through periodontal erosion to form recurrent transient bacteremia, leading to chronic systemic inflammation

and allowing bacteria to enter the central nervous system [30]. Ingar [31] et al. proposed that the peptidyl arginine deiminase secreted by *P. gingivalis* affects the occurrence and development of rheumatoid arthritis and AD through the citrullination process. Our research group established the *P. gingivalis* peripheral infection model and explored the relationship between *P. gingivalis* peripheral infection and AD. The results showed that *P. gingivalis* 16S rDNA was not detected in brain tissues after 4 weeks and 12 weeks of *P. gingivalis* infection, but *P. gingivalis* infection caused a high expression of inflammatory factors in the circulatory systems and hippocampal tissues of rats, such as IL-1β and TNF-α. Meanwhile, Tau hyperphosphorylation and the deposition of β-amyloid protein-42 (Aβ-42) in rat hippocampal tissue were detected. The above results suggest that *P. gingivalis* peripheral infection can promote the occurrence and development of AD-related pathological changes through the inflammatory response. Neuron loss caused by apoptosis is one of the pathological features of AD. Therefore, this study takes neuron apoptosis as the entry point to explore the influence of *P. gingivalis* peripheral infection on neuronal apoptosis and its possible mechanism.

Materials and methods

P. gingivalis bacterial suspension preparation

P. gingivalis (ATCC strain 33277) was obtained from the State Key Laboratory of Oral Diseases at Sichuan University (Chengdu, China). The resuscitated strains were inoculated in a brain-heart infusion liquid medium containing sterile defibrated sheep blood, vitamin K1 (10 mg/mL) and heme chloride (0.5 mg/mL). The strain was grown under anaerobic conditions (85% N_2, 10% H_2 and 5% CO_2) at 37°C. Harvested bacteria at the log phase and were centrifuged for 5 min (12000 rpm, 4°C) and the precipitate was resuspended in sterile PBS; these steps were repeated twice. The bacterial suspension was adjusted by a Maxwell turbidimeter (BD, USA) to a final concentration of 10^8 CFU/mL [32].

Animal treatment

Three-month-old (200 ± 10 g) male Sprague-Dawley rats (Grade: SPF) were supplied by the Experiment Animal Center of Sichuan University and were fed in the State Key Laboratory of Oral Diseases of Sichuan University. The rats were kept at 22 ± 2°C on daily 12 h light-dark cycles and were allowed free eating and drinking. All of the rats (n=60) were randomly divided into four groups: a 4-week experimental group, a 4-week control group, a 12-week experimental group and a 12-week control group (15 rats in each group). The experimental group was injected with the *P. gingivalis* bacterial suspension in the tail vein (three times a week, 200 μl, 10^8 CFU/ml). The control group was injected with an equal amount of PBS. Before and after the injections, the animals were sterilized with medical iodine, and the bleeding was completely stopped after the injection to prevent wound infection. The animals were confirmed to have no clinical symptoms of acute infection or death. The samples were taken 24 h after the last injection.

A subgroup of the rats (n=6 in each group) were deeply anesthetized with an intraperitoneal injection of chloral hydrate (the concentration was 6%) and then fixed by transcardial perfusion with 0.9% NaCl followed by 4% paraformaldehyde. After perfusion, the brain tissue was separated and placed in a 4% paraformaldehyde solution for approximately 48-72 h. After paraffin embedding the brain tissue, coronal sections of the brain were cut 30 μm thick for Nissl staining and hematoxylin & eosin (HE) staining and 5 μm thick for the TUNEL method. The remaining rats in each group were anesthetized intraperitoneally, and fresh hippocampal tissue was isolated. Some of the tissue was frozen for Western blot, and the remaining fresh hippocampal tissue was used for flow cytometry to detect intracellular calcium ion concentrations in the hippocampal area.

Nissl staining

Paraffin sections were dewaxed and soaked in preheated 1% toluidine blue solution for 20 min. After gradient dehydration, the samples were washed using xylene, placed under cover slips and analyzed under a microscope (BA400Digital, MOTIC, China), and the images were analyzed using Image Pro Plus 6.0 software (Media Cybernetics, USA).

TUNEL method

The apoptosis index in the rat hippocampal area was determined using the TUNEL method.

Paraffin sections of approximately 5 μm thickness were made, and the procedures were strictly applied in accordance with the kit's requirements. Six sections of each paraffin block were acquired, and 4 fields of view were randomly selected. The color of the apoptotic nucleus was light yellow or brownish yellow, and the negative expression was blue. The images were analyzed using Image Pro Plus 6.0 software (Media Cybernetics, USA). Apoptosis index calculation formula: number of apoptotic cells/total number of observed cells ×100%.

Western blot

Rat hippocampal tissue was ground with liquid nitrogen, and protein lysate was added. After lysis for 30 min, the supernatant was obtained, and the protein concentration was determined using the BCA method. First, the supernatant was stored at -80°C. Each group of samples was dose-normalized to 30 μg and transferred to PVDF membranes by SDS-PAGE. Then, the PVDF membrane was soaked in a skim milk powder solution and sealed for 2 h (37°C). Primary antibody (Santa Cruz, USA) at a dilution of 1:300 was added overnight at 4°C. TBST was used to wash the membrane 3 times for 10 min each. After that, we added the secondary antibody (Santa Cruz, USA) at a dilution of 1:2000 for 1 h (37°C) and again washed with TBST 3 times. β-actin was used as an internal reference, and the ratio result indicates the relative expression level of each target protein.

Intracellular calcium concentration

The fresh hippocampal tissue was gently milled and filtered on a 70 μm cellular sieve. The filtrate was filtered using a 40 μm cellular sieve and centrifuged at 1000 rpm for 5 min. The precipitate was resuspended with Dulbecco's phosphate buffered saline (D-PBS) and centrifuged at 1000 rpm for 5 min; these steps were repeated twice. Fluo-4 AM was diluted with D-PBS to a concentration of 4 μM/ml. In dark conditions, the cell precipitate was resuspended with 300 μL of Fluo-4 AM diluent and incubated for 50 min at 37°C. The cells were washed three times with D-PBS and incubated at room temperature (22°C) for 30 min to ensure the Fluo-4 AM was fully transformed into Fluo-4. The fluorescence intensity was determined using flow cytometry. The excitation wavelength was 488 nm, the emission wavelength was 525 nm, and the fluorescence intensity reflected the intracellular calcium ion concentration.

Statistical analysis

The data were analyzed with SPSS software, version 19.0 (SPSS Inc.) and are presented as the means ± standard deviations. One-way ANOVA was performed, and P values <0.05 were considered statistically significant.

Results

The number of Nissl bodies decreased

Compared with the control group, the number of Nissl bodies in the experimental group decreased, and the cells were loosely arranged after *P. gingivalis* infection. There was no significant difference between the experimental group and the control group after 4 weeks of infection (P>0.05). The difference in the number of Nissl bodies between the control group and the 12-week group was statistically significant (P<0.05), and there was also a statistically significant difference between the number of Nissl bodies in the 4-week and 12-week subgroups in the experimental group (P<0.01) (**Figure 1**).

Hematoxylin & eosin staining

HE staining (**Figure 2**) showed that there was no significant difference between the experimental group and the control group after 4 weeks of infection. In the 12-week group, there was vertebral cell degeneration, nuclear pyknosis and hyperchromatism, and the number of cells decreased. No obvious abnormal lesions were observed in the control group, and the difference between the two groups was statistically significant. The lesions were more severe in the 12-week group than in the 4-week group, suggesting that *P. gingivalis* infection could lead to cell damage in the hippocampi of SD rats.

P. gingivalis infection affects cell apoptosis

TUNEL staining (**Figure 3**) showed that there were few apoptotic cells in the hippocampi of the control group, and obvious apoptosis was detected in the hippocampi of the experimental group. The above changes were significantly different between the experimental group and the control group at 4 weeks and 12 weeks

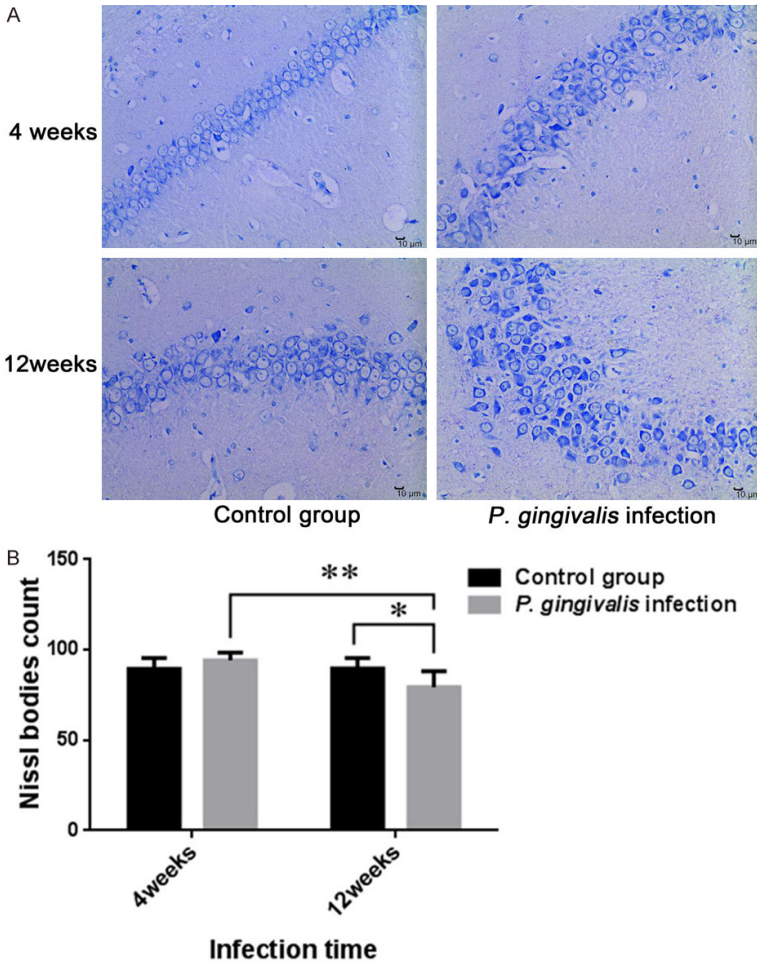

Figure 1. Results of Nissl staining in SD rat hippocampal tissue. A. Nissl staining picture (400×). B. The number of Nissl bodies in each group (*P<0.05, **P<0.01).

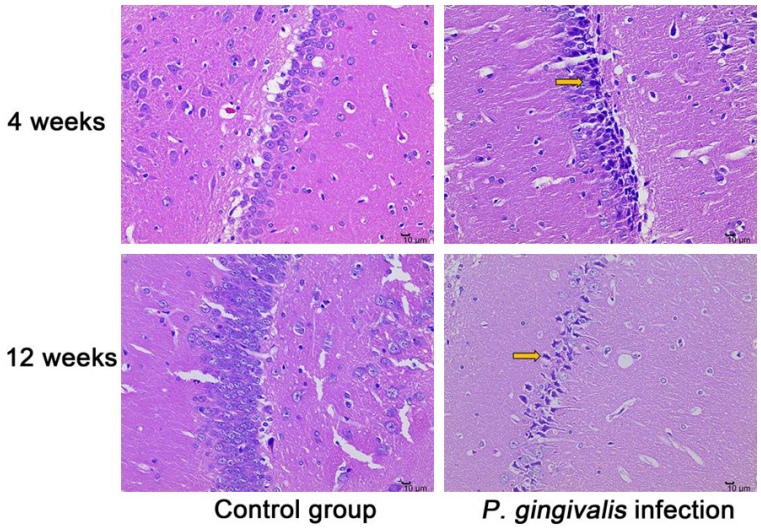

Figure 2. The results of HE staining in the hippocampal tissue of SD rats (400×).

(P<0.05). The difference between the experimental group at 4 weeks and 12 weeks was also statistically significant (P<0.01). The results showed that *P. gingivalis* infection could promote apoptosis in the hippocampal tissue of SD rats in a time-dependent manner.

Cleaved caspase-3, PSD-95 and NR2B expression

Cleaved caspase-3 (P<0.01) (**Figure 4**) and PSD-95 (P< 0.05) (**Figure 5**) exhibited increased expression after 4 weeks of infection compared with the expressions in the control group. Although NR2B (**Figure 5**) expression was increased, it was not a statistically significant difference. After 12 weeks of infection, the expressions of cleaved caspase-3 (P<0.01) and NR2B were increased (P<0.01), and the expression level of PSD-95 had increased significantly (P< 0.001). In the experimental group, the changes in the expressions of cleaved caspase-3 (P<0.05) and NR2B (P<0.05) were significantly different between the 4- and 12-week subgroups.

Intracellular calcium concentration was increased in the hippocampus

The intracellular calcium concentration in the hippocampal cells of the experimental group increased compared with that of the control group, but there was no significant difference after 4 weeks of infection, while the concentration increased significantly in the experimental group after 12 weeks of infection (P<0.01). Additionally, there was a statistically significant difference between the 4- and 12-week

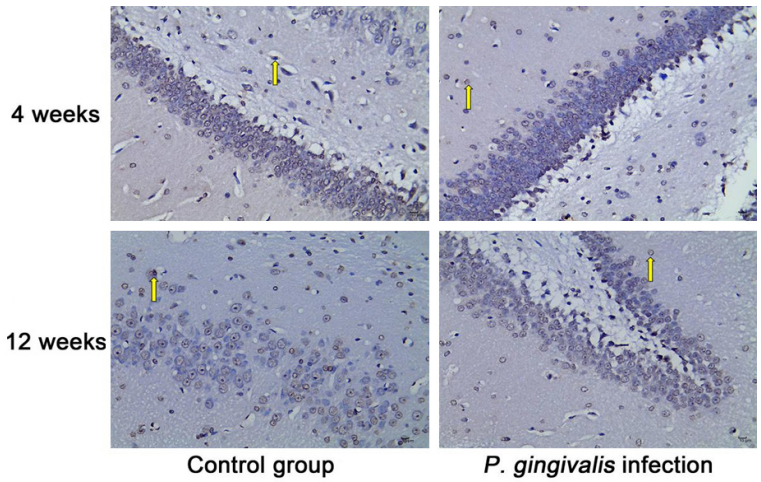

Figure 3. The results of TUNEL staining in the hippocampal tissue of SD rats (400×).

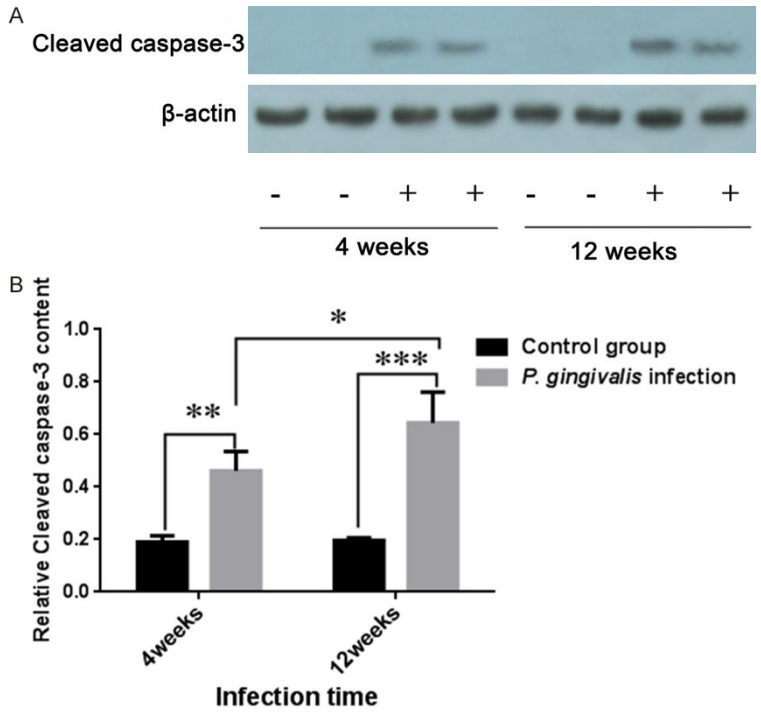

Figure 4. The expression of Cleaved caspase-3 in the hippocampal area of SD rats was increased after *P. gingivalis* infection. A. Western blot results of Cleaved caspase-3 in hippocampal cells. B. The expression of Cleaved caspase-3 in each group (*P<0.05, **P<0.01).

subgroups of the experimental group (P<0.05) (**Figure 6**).

Discussion

Here, we demonstrated that *P. gingivalis* infection can promote the apoptosis of cells in the hippocampus of SD rats through excitatory neurotoxicity, which may provide a basis for the prevention and treatment of periodontitis to slow the occurrence and development of AD.

AD is the most common type of dementia, and its mechanisms remain unclear. In recent years, an increasing number of researchers have demonstrated that periodontitis is closely related to AD. However, the exact molecular mechanism is unclear [2]. Neuron loss due to apoptosis is closely related to AD. Therefore, we selected wild-type rats as subjects in this study to explore the effect and mechanisms of *P. gingivalis* on the apoptosis of hippocampal cells in SD rats.

Nissl bodies are an important morphological index to show the functional activity of neurons. The existence and change in the quantity of Nissl bodies are important indicators of whether neurons are damaged. In this experiment, we used Nissl staining to show that the number of Nissl bodies in the hippocampus of SD rats after *P. gingivalis* infection decreased, suggesting that neurons in this area were damaged. At the same time, HE staining showed that *P. gingivalis* venous infection leads to changes in cell morphology in the hippocampus of SD rats, resulting in cell degeneration and reduction and red, hyperchromatic nuclei. Therefore, we speculated that *P. gingivalis* infection may promote apoptosis and necrosis of cells in this area, further resulting in neuron loss. To clarify the above speculation, we tested the expression of cleaved caspase-3 in the hippocampus. Cleaved caspase-3 is an activated form of caspase-3, which is the core protein in the apoptosis cascade and the executor of the cell apoptosis process [33]. The results showed that the

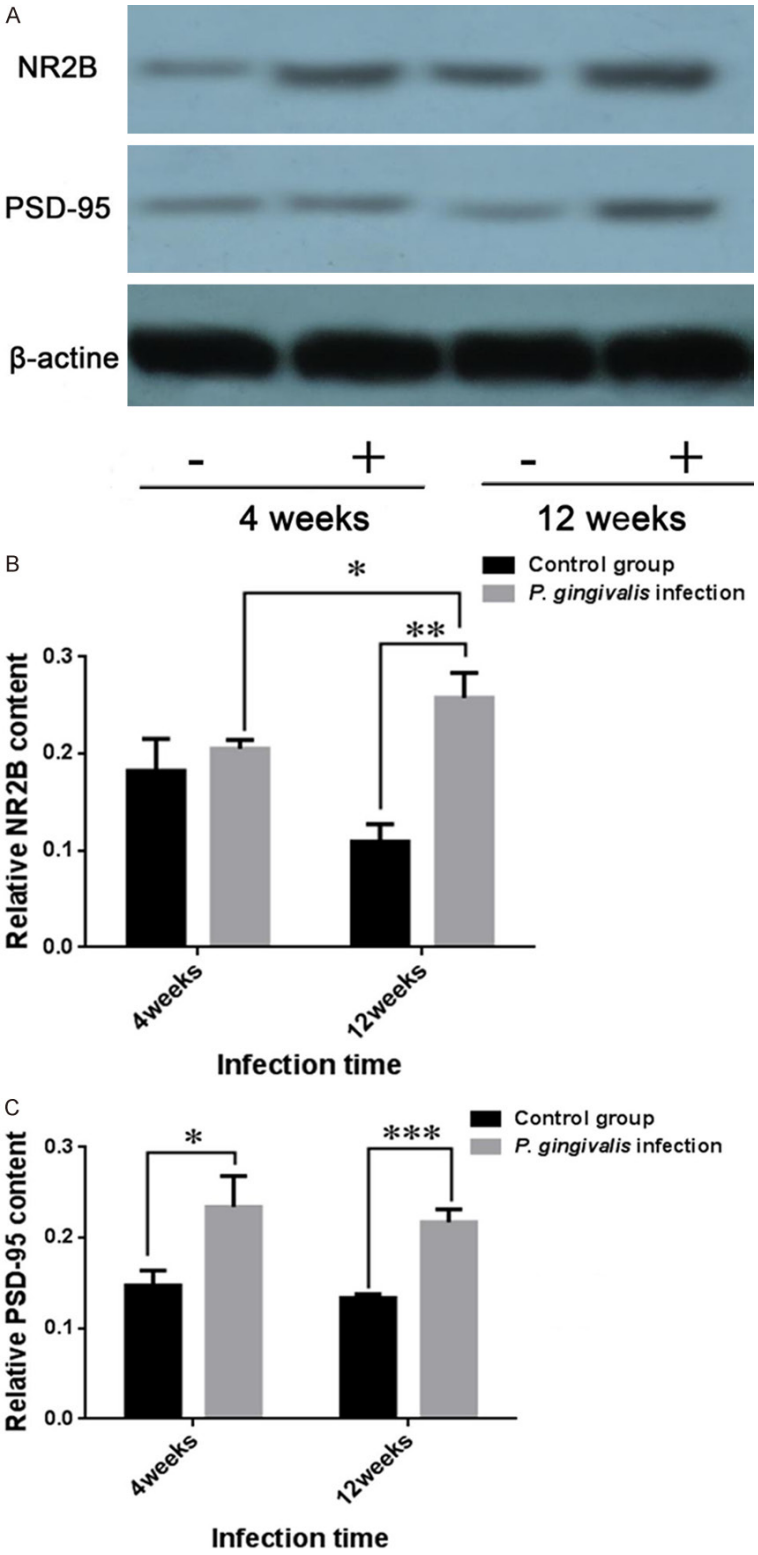

Figure 5. The expressions of NR2B and PSD-95 in the hippocampal area of SD rats were increased after *P. gingivalis* infection. A. Western blot results of NR2B and PSD-95 in hippocampal cells. B. The expression of NR2B in each group. C. The expression of PSD-95 in each group (**P*<0.05, ***P*<0.01, ****P*<0.001).

expression of cleaved caspase-3 increased in the hippocampi of the SD rats after *P. gingivalis* venous infection in a time-dependent manner. The experimental results showed that after intravenous infection by *P. gingivalis*, there was cell apoptosis in the hippocampi of the rats.

Intracellular calcium overload caused by excitatory amino acid toxicity can induce apoptosis, which is one of the mechanisms of apoptosis in brain tissue and is associated with a variety of neurodegenerative diseases, including AD. Increased glutamate release and its receptor expression and activation can all lead to excitatory amino acid toxicity, resulting in neuronal damage. Therefore, this study explored whether intravenous infection by *P. gingivalis* might induce neuronal apoptosis through excitatory amino acid toxicity. NMDAR is the main glutamate receptor in brain tissue, which is widely distributed in brain tissue and closely related to excitatory amino acid toxicity. The NMDAR subunit NR2B expression and intracellular calcium ion concentration in the hippocampal area of SD rats were measured. The results showed that NR2B expression increased in a time-dependent manner after *P. gingivalis* infection. Meanwhile, the intracellular calcium ion concentration increased, which was consistent with the increased expression of NR2B. The above results are the same as those of Yun yang et al. [34]. Furthermore, our experiments also found that the expression of PSD-95 in the hippocampal area of SD rats was increased. PSD-95

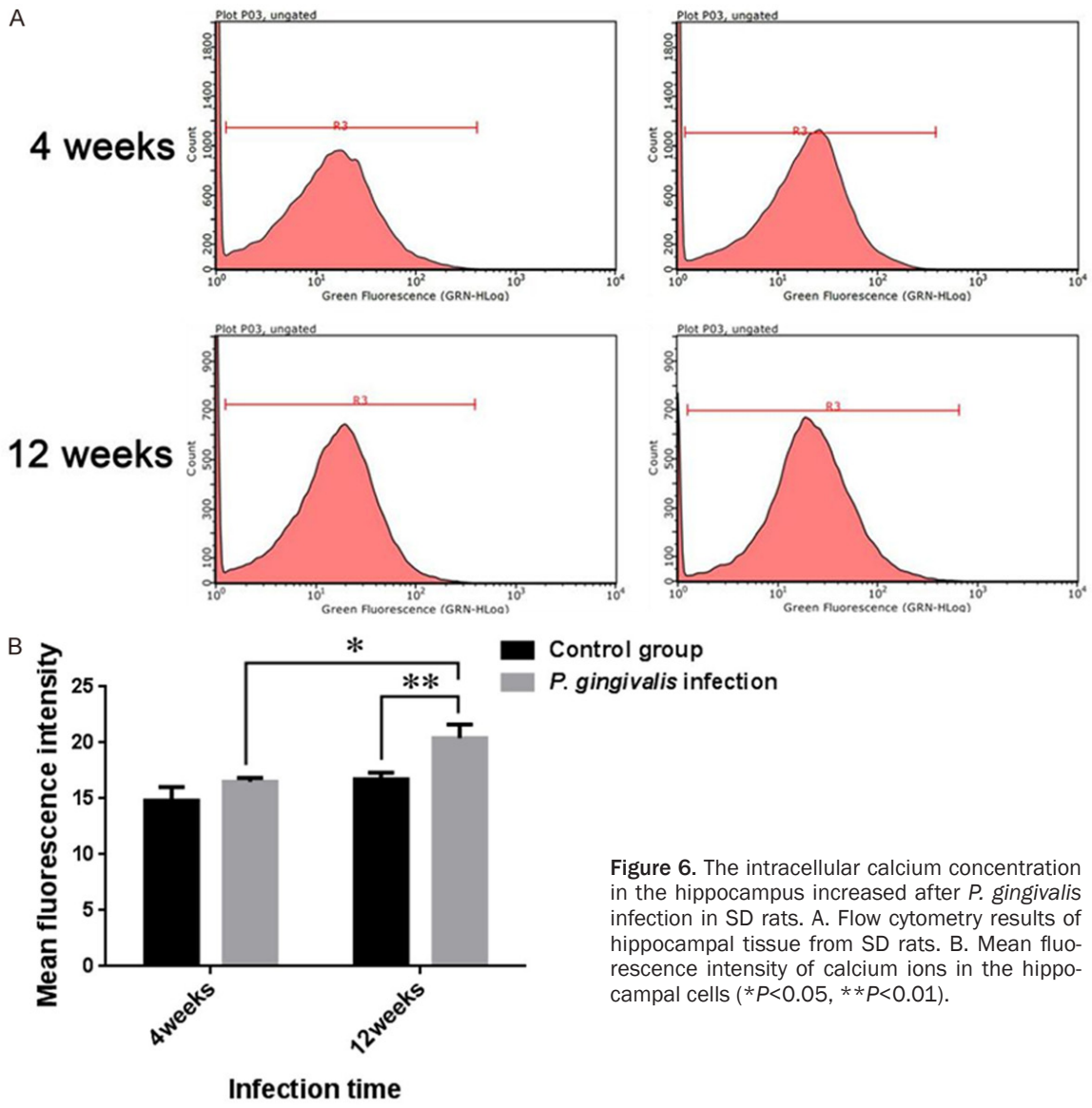

Figure 6. The intracellular calcium concentration in the hippocampus increased after *P. gingivalis* infection in SD rats. A. Flow cytometry results of hippocampal tissue from SD rats. B. Mean fluorescence intensity of calcium ions in the hippocampal cells (**P*<0.05, ***P*<0.01).

can enhance the function of NMDAR by coupling with NR2B through a protein binding domain and can also participate in the process of neuronal apoptosis in brain tissue. In this study, we found that the expression of PSD-95 increased after *P. gingivalis* infection, which was consistent with the change in NR2B, suggesting that PSD-95 promotes intracellular calcium flow in coordination with NR2B.

Thus far, our study has found that a venous infection with the mainly periodontal pathogenic bacteria, *P. gingivalis*, can promote the expression of the NMDAR subunit NR2B and PSD-95 in the hippocampal region, causing intracellular calcium overload and leading to apoptosis. These changes may be linked to inflammation. Inflammation is associated with AD, which can cause neuronal apoptosis. Research has found that quercetin promotes neuronal and behavioral recovery by suppressing inflammatory responses and apoptosis in a rat model of intracerebral hemorrhage [35]. Some researchers have found that free radicals form when sulfur dioxide is inhaled, causing oxidative damage in the brain and leading to inflammatory responses. These inflammatory reactions can upregulate the expression of NMDAR to high levels. Activated NMDAR channels lead to an increased calcium ion concentration in cells, and calcium overload promotes neuronal apoptosis. Furthermore, inflammation

can promote the expression of PSD-95, increasing the postsynaptic membrane thickness and affecting synaptic plasticity [36, 37]. These studies further revealed that inflammation may affect AD by promoting the apoptosis of neurons and that this may be related to the NMDA receptor and PSD-95. Periodontitis can lead not only to local periodontal tissue infection but also to the translocation of periodontal pathogens, bacterial products, toxins and inflammatory products in the circulatory system, as well as the colonization of other organs. In previous studies, the research team found that *P. gingivalis* intravenous infection can cause brain tissue inflammation; however, the relationship between inflammation and the above mechanisms needs to be studied further.

In summary, *P. gingivalis* can lead to the apoptosis of hippocampal cells in SD rats after an intravenous infection, which may promote the occurrence and development of AD.

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Disclosure of conflict of interest

None.

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