STAT3 signaling pathway could be involved in the progress of cognitive dysfunction caused by ligature-induced periodontitis

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Research

Keywords: ligature-induced periodontitis, cognitive function, neuroinflammation; STAT3 signaling pathway, APP processing, Alzheimer's disease

Posted Date: February 13th, 2020

DOI: https://doi.org/10.21203/rs.2.23488/v1

Abstract

Background: Increasing evidence suggests a causal link between periodontitis and Alzheimer's disease (AD). Systemic inflammation initiated by periodontitis may mediate the development of AD. Our study aims to investigate the effect of ligature-induced periodontitis on cognitive function and the role of STAT3 in this process.

Materials and Methods: Ligature-induced periodontitis was established, and the rats were treated intraperitoneally with/without the pSTAT3 inhibitor cryptotanshinone (CTS, 5 mg/kg). Alveolar bone resorption and periodontal inflammation were detected by micro-computed tomography (micro-CT) analysis and histopathological evaluation. The expression of cytokines (IL-1β, IL-6, IL-8, IL-21) in the periphery was evaluated by RT-PCR and ELISA. Locomotor activity and cognitive function were evaluated by the open field test and the Morris water maze test, respectively. The activation of microglia and astrocytes was assessed by immunohistochemistry. In the cortex, the cytokines mentioned above were also detected, while the activation of the STAT3 signalling pathway (JAK2, STAT3, and pSTAT3) was assessed by Western blotting. The expression of amyloid precursor protein (APP) and its key secretase enzymes was evaluated by RT-PCR, while the total Aβ and the ratio of Aβ1-40/1-42 were measured via ELISA.

Results: In periodontal ligature rats, significant alveolar bone resorption and local inflammatory cell infiltration were present. Apparent increases in inflammatory cytokines (IL-1β, IL-6, IL-8 and IL-21) were detected; this change was relieved by the pSTAT3 inhibitor CTS. Additionally, spatial learning and memory function was impaired, while locomotor activity was not affected. Activated microglia and astrocytes were found in the cortex and hippocampus. In the cortex, these inflammatory cytokines showed similar changes when the STAT3 signalling pathway was activated. The processing of APP by β- and γ-secretases was promoted, and both the total Aβ and the ratio of Aβ1-40/1-42 were upregulated. Changes in the brain were also attenuated by CTS.

Conclusions: Ligature-induced periodontitis in rats resulted in systemic inflammation and further impairments in cognitive function, leading to AD-like pathology. The activation of the STAT3 signalling pathway may play an important role in this process by increasing inflammatory load in both the periphery and brain, promoting neuroinflammation and modulating APP processing. This study may provide novel insights into periodontal intervention strategies and potential targets for AD prevention.

Introduction

Periodontitis, a potentially transmissible chronic infection caused by plaque biofilm, has resulted in one or more instances of damage to the peridontium in 70% of the global population [1]. As a product of interactions among bacterial pathogens, including their toxic factors, and host inflammatory responses, periodontitis has a multifactorial aetiology with a marked inflammatory profile [2]. Chronic recurrent periodontal inflammation leads to the destruction of the tooth support apparatus, and there is also

increasing evidence that periodontal disease is an important risk factor for systemic diseases such as cardiovascular disease and diabetes and reduced respiratory function [3-5].

With the accelerating trend of population aging, the detriment of cognitive dysfunction diseases to human health has become increasingly prominent. Alzheimer's disease (AD) is a progressive neurodegenerative disease with a slow and irreversible course, and the clinical manifestations of AD include gradual cognitive dysfunction and psychosis [6-7]. AD is characterized by the formation of extracellular amyloid β peptide (Aβ) deposits and the subsequent intracellular accumulation of neurofibrillary tangles consisting of abnormally phosphorylated tau [8]. Several attempts have been made to treat AD using anti-amyloid strategies with disappointing results. Recently, the "inflammation hypothesis of AD" has become a topic of debate [9].

Currently, investigation regarding the links between periodontitis and cognitive dysfunction are still at the stage of epidemiological and clinical case-control studies, but few of these studies have clarified the underlying pathology. Wu et al. [10] reported that the systematic administration of lipopolysaccharide can upregulate the expression of amyloid precursor protein (APP) in the brain and promote the development of AD. Ilievski et al. [11] detected neurodegeneration and the formation of extracellular Aβ1-42 after the oral application of *Porphyromonas gingivalis* (*P. gingivalis*). In a previous study, we also demonstrated that the intraperitoneal injection of *P. gingivalis*-lipopolysaccharide (*P. gingivalis*-LPS) can cause cognitive dysfunction associated with brain inflammation via the NF-κB/TLR4 signalling pathway [12]. It is well known that periodontitis is initiated by complex biofilms containing several likely periodontal pathogens. A limitation of the above reports is the use of similar periodontitis models established by *P. gingivalis* application, a specific periodontal pathogen. It is necessary to develop other models, such as ligature-induced models, which are more likely to mimic clinical periodontitis and have been commonly applied [13]. However, the effect of ligature-induced periodontitis on cognitive function has not yet been investigated.

Periodontitis is an inflammatory condition that can lead to chronic systemic inflammation and high levels of systemic cytokines. It shares epidemiological associations and underlying inflammatory mechanisms with systemic chronic inflammatory disease [13]. Common inflammatory cytokines have been shown to be increased in patients with chronic periodontitis and are capable of initiating and maintaining mechanisms associated with the development of chronic systemic diseases [14]. Signal transducers and activators of transcription (STATs) are one of the major regulatory elements in the production of inflammatory cytokines and signalling molecules [15]. Among them, STAT3 is critical for regulating the expression of cytokines, chemokines and other mediators that can induce and maintain an inflammatory environment [16]. Both its role in periodontitis and its impact on cognitive function remain unknown.

In the present study, we induced periodontitis in SD rats by placing ligatures at the maxillary first molars and evaluated systemic inflammatory load as well as AD-like pathology. Periodontal inflammation and bone resorption similar to periodontitis were indicated by micro-CT and HE staining. The relative expression of interleukins in the periphery was detected by RT-PCR and ELISA. The effects of ligature-

induced periodontitis on cognitive function were evaluated by the MWM test. The levels of interleukins and the activation of microglia and astrocytes in the brain were observed by RT-PCR, ELISA and immunohistochemistry. To further analyse the underlying mechanism, the activation of STAT3 and the processing of APP were assessed by RT-PCR, ELISA and Western blotting.

Materials And Methods

2.1 Animals

All experimental protocols were approved by the ethical committee of the Animal Care and Experimental Committee of Shanghai Jiao Tong University School of Medicine and were performed according to the guidelines from the EU Directive 2010/63/EU. Efforts were made to minimize suffering due to surgery and to reduce the overall number of animals we used.

Ten-week-old male SD rats of Specific pathogen-free (SPF) grade were provided by the Shanghai SIPPR-BK Laboratory Animal Co., Ltd. Standard housing of temperature (18-22℃) and humidity (55-65%) with a 12-h light/dark cycle and free access to food and water was maintained during the experimental period. 32 rats were randomly housed 2 per cage and divided into four groups: control, CP, CP plus CTS and CTS group. After anesthesia (intraperitoneal injection, 0.8ml 5% chloral hydrate per 100g weight), the ligature of maxillary first molars in the CP group and CP plus CTS group was administrated. In addition, the rats in the CP plus CTS group were injected with CTS (5mg/kg, i.p.) 1 hour in advance. The control group was given an equivalent volume of saline. The amount of CTS was calculated based on earlier studies [17]. Three days after the last administration, behavioral tests were performed to evaluate the cognitive function of the rats. Timeline and intraoral photographs could be found in Supplementary Figure 1-2.

The 0.25mm ligature wire was provided by department of orthodontics, Shanghai Ninth People's Hospital. CTS was purchased from Abmole (USA) and dissolved according to the instructions from Selleck (Shanghai, China) (2% DMSO+30% PEG300+5% Tween 80+ddH_2O).

2.2 Measurement of Alveolar Bone Resorptionby Micro-Computed Tomography (CT)

The maxillae of rats were obtained to detect bone parameters by micro-CT. Fixed in 4% paraformaldehyde, the bone morphometry was assessed using Skyscan1172 (Bruker, Kontich, Belgium) with an accuracy of 18μm. Parameters including bone volume percentage (bone volume/total volume, BV/TV), Bone surface/volume ratio (BS/BV) and Bone mineral density (BMD) were calculated.

2.3 Assessment of periodontal inflammation responses by Histologic Staining Assay (HE staining)

The maxillae of rats were immersed with EDTA decalcifying solution (10% EDTA-2Na) and replaced every 5 days for 4-6 weeks for further histologic staining assay. After decalcification, hematoxylin-eosin (HE) staining assay was used to detect the inflammation response. Samples were then dehydrated through a serial alcohol gradient and embedded in paraffin wax blocks. Before immunostaining, 5-μm-

thick tissue sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS. Then sections were stained with hematoxylin and eosin (H&E). After that, sections were dehydrated through increasing concentrations of ethanol and xylene.

2.4 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood from rats (5 ml per rat) was collected from abdominal aorta in the presence of heparin as the anticoagulant. 3 ml of the whole blood was diluted with sterile PBS of the same volume and gently resuspended. 6 ml of the diluted whole blood fraction was overlaid onto 3 ml of the Ficoll-Paque Plus (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and then subjected to 800g for 20 mins at RT with the centrifuge brake “off”. Then PBMCs layers were washed twice with RPMI 1640 media by centrifugation at 1200rpm for 5 mins at 4℃. After isolation, all samples were dissolved in Trizol reagent (Takara, Kusatsu, Shiga, Japan) for lysis of cells to extract RNA.

2.5 RNA extraction and RT-PCR analysis

The RNA was extracted from PBMCs and homogenization of cortex using Trizol reagent (Takara, Kusatsu, Shiga, Japan) and the Total RNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) respectively. The purity and concentration of RNA, as well as the cDNA synthesis, were conducted according to Li et al. [18]. Briefly, the purity and concentration were measured by a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). The ratios of the absorbances at wavelengths of 260 and 280 nm were1.8–2.0. Subsequently, an RT-PCR assay was performed using SYBR Premix Ex Taq^{TM} (Takara, Kusatsu, Shiga, Japan) on a Roche LightCycler 480 Real-Time PCR Detection System (Roche, Basel, Switzerland) according to the manufacturer’s protocol. Data were then processed using the $2^{-\Delta\Delta CT}$ method. All results were based on at least three independent tests, and the final results were expressed as normalized fold values relative to the control group. The sequences of genes including Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), interleukin-1β (IL-1β), IL-6, IL-6R, IL-8, IL-21, IL-21R, APP, amyloid precursor-like protein 1 (APLP1), APLP2, a disintegrin and metalloproteinase 10 (ADAM10), ADAM17, β-site APP cleaving enzyme 1 (BACE1), presenilin 1 (PS1), PS2 and their primer pairs were listed in table 1.

2.6 Protein levels in plasma and cortex measured by ELISA

Of the approximately 5ml of blood collected from rats, 2ml of blood were collected in heparinized tubes for the measurement of plasma cytokines. After centrifuging (4℃, 2500rpm*15min), plasma was immediately aliquoted into 1.5 milliliter cryogenic tubes and frozen at −80°C until use. For tissue, radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Beijing, China), 1% protease inhibitor cocktail (Sigma, St. Louis, MO, USA), and 1% PMSF (Beyotime, Beijing, China) were used to homogenize samples of the cerebral cortex. Protein qualification was performed by BCA Protein Assay Kit (Beyotime, Beijing, China). All ELISA kits were rat specific. Equal amounts of protein were used in ELISA to measure levels of IL-1β (detection limits<0.1pg/ml; UBI, Sunnyvale, CA, USA), IL-6 (detection limits<0.1pg/ml; UBI, Sunnyvale, CA, USA), IL-8 (detection limits<0.1pg/ml; UBI, Sunnyvale, CA, USA), IL-21 (detection limits<0.1pg/ml; UBI, Sunnyvale, CA, USA), total Aβ (detection limits<1.0ng/ml; UBI, Sunnyvale, CA, USA),

Aβ1-40 (detection limits<1.0ng/ml; Enzyme-linked Biotechnology, Shanghai, China) and Aβ1-42 (detection limits<0.1ng/ml; Enzyme-linked Biotechnology, Shanghai, China) both in the cortex and plasma according to the manufacturer's instructions.

2.7 Open filed test

The open field in the present study consisted of a rectangular arena (530mm×478mm), enclosed by a black wall, 590mm in height (Mobile Datum, Shanghai, China). The test was initiated by gently placing a single rat in the middle of the arena, allowing the animal to move freely for 5 minutes while being recorded.

2.8 Morris water maze (MWM) test

The MWM test was conducted in a round pool 160 cm in diameter and 55 cm in depth (Mobile Datum, Shanghai, China). The pool was filled with water made opaque with white non-toxic water-based tempura paint. The water temperature was controlled to remain with a range equivalent to that of room temperature (22±1°C). The platform was placed in the center of one quadrant of the pool and submerged 2.5 cm beneath the water surface; it remained in the same position throughout the learning trials and was removed from the pool during the probe test. A video-tracking system (Shanghai Jiliang Software Technology Co., Ltd.) was used to monitor and record the swimming activity of the rats. The rats should have learned to use the visual tips around the pool to find the hidden platform within 90s, otherwise it would be gently guided to the platform and allowed to re-orient for an additional 10s. Each rat was trained four times per day with a 30s of rest per training interval. To examine spatial reference memory, a probe test was carried out on the sixth day when the platform was removed from the pool and each rat was placed into the water at the two quadrants furthest from the platform used on days 1–5, being allowed to navigate freely for 60s.

2.9 Western blot

The samples of the cerebral cortex of rats in four groups were homogenized and lysed by RIPA containing 1% protease inhibitor cocktail and 1% PMSF (Beyotime, Shanghai, China). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred onto PVDF membrane blocked with 5% skimmed milk as previously described [18]. A pre-stained protein marker (Thermo Fisher Scientific, MA, USA) was run in parallel to detect the molecular weight of proteins. GAPDH was used as a protein loading control according to its high and constant expression in most tissues and cell types, earning the gene and its protein housekeeping status. Proteins were probed with appropriate antibodies including anti-JAK2 (Rabbit mAb, 1:500, 3230T; Cell Signaling Technology, USA), anti-STAT3 (Rabbit mAb, 1:1000, 4904T; Cell Signaling Technology, USA), anti-phosphor STAT3-Tyr705 (Rabbit mAb, 1:1000, 9145S; Cell Signaling Technology, USA) and anti-GAPDH (Rabbit mAb, 1:1000, AB-P-R001, Goodhere Biotechnology Co., Hangzhou, China). The data were quantified by Image J 1.51j8.

2.10 Immunohistochemistry

Rats were anesthetized with 10% chloral hydrate and perfused with cool PBS before removal of the brain.

One hemisphere was placed in 4% paraformaldehyde overnight at 4°C, after which paraffin sections were prepared. This procedure is consistent with the previous study [12]. Briefly, brain sections were incubated with 3% H_2O_2 in methanol, blocked with 10% goat serum and incubated overnight at 4 °C with the following primary antibodies: Iba1 (Goat pAb, 1:400, ARG63338; Arigo Biolaboratories, Hsinchu City, Taiwan, China) and GFAP (Rabbit pAb, 1:400, ab7260; Abcam) to label microglia and astrocytes. After being washed, sections were incubated with biotinylated goat anti-rabbit or goat secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). After being rinsed with PBS, streptavidin-labeled peroxidase was added and left for 30 min. This was followed by further rinsing, after which newly prepared 3,3'-diaminobenzidine (DAB) solution was added, and the mixture was left for the reaction to develop. The sections were dyed with hematoxylin and dipped in 1% hydrochloric acid in alcohol for differentiation. They were then washed in ammonia and stained blue, after which they were rinsed with water.

For cell counting, images were obtained with a Leica camera. The numbers of Iba1-positive or GFAP-positive cells were determined by counting positive cells in two areas of each section in every tenth serial coronal section. At least three sections were analyzed per rat, and the average of the individual measurements was used to calculate group means.

2.11 Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). P values were calculated with one-way ANOVA and two-way ANOVA with the GraphPad Prism software 7.0. An analysis of variance was performed using Turkey's post hoc multiple comparison test. A value of $p<0.05$ was indicative of statistical significance.

Results

3.1 Assessment of alveolar bone resorption and periodontal inflammation

As shown in Figure 1A, posterior maxillary bone loss was observed in the CP group. BV/TV and BMD were decreased while BS/BV was increased in the CP group and was reversed by CTS (Fig. 1B-D). Further HE staining suggested that periodontal ligature led to a significant decrease in alveolar bone height, irregular bone surface and infiltration of inflammatory cells (Fig. 1E). All of these changes, which were alleviated by CTS, suggested that periodontal inflammation and bone resorption similar to periodontitis were induced by periodontal ligature.

3.2 Effects of ligature-induced periodontitis on the level of interleukins in the peripheral blood

RT-PCR assays were performed to evaluate the mRNA expression of IL-1β, IL-6, IL-6R, IL-8, IL-21 and IL-21R in PBMCs (Fig. 2). The protein levels of IL-1β, IL-6, IL-8 and IL-21 in the plasma were detected by ELISA (Fig.3). The CP group showed an approximately threefold increase in the mRNA expression of IL-21 and IL-21R and a twofold increase in the mRNA expression of IL-6 and IL-6R, and these increases were

reversed by CTS. Upregulated expression of inflammatory factors was induced by ligature-induced periodontitis and significantly prevented by CTS.

3.3 Effects of ligature-induced periodontitis on locomotor activity

The open field test was used to assess whether periodontal ligature or the administration of CTS affects the spontaneous activity of rats. Behaviour parameters, including total distance covered (Fig. 4A), time of rest (Fig. 4B) and average speed (Fig. 4C), were not significantly different among the groups. Behavioural performance indicated that the locomotor activity of the rats was not affected by periodontal ligature or CTS.

3.4 Effects of ligature-induced periodontitis on spatial learning and memory

The Morris water maze was conducted to assess whether periodontal ligature affects the learning and memory of rats. Over the 5-day training period, the latency of all groups decreased chronologically (Fig. 5A). The escape latency of the CP group was evidently longer than that of the control group after the 5-day training period. No significant differences were observed between the control group and the CP plus CTS group. The platform was removed on the sixth day, and the following parameters were assessed in the probe test: the number of platform crosses in the target quadrant and the percentage of time spent in the target quadrant. Both of these parameters which were significantly reduced in the CP group (Fig. 5B-C). During the probe test, the rats in the control group learned to navigate directly to the quadrant that had contained the hidden platform. However, this behaviour was strikingly compromised in the CP group (Fig. 5D). The abovementioned changes in the CP group were reversed by CTS. The results of the behavioural tests revealed that periodontitis may be an important risk factor for cognitive dysfunction.

3.5 Effects of ligature-induced periodontitis on microglia and astrocytes

As shown in Figure 6, microglia and astrocytes were observed to be activated in the CP group. Activated microglia, characterized by irregular protrusions and increased cell body volume, were positively stained for ionized calcium-binding adaptor molecule 1 (Iba1). Activated microglia were observed in the hippocampus and cortex of the CP group, whereas activated microglia were rarely observed in the control group (Fig.6A, C-D). Activated astrocytes were positively stained for GFAP, and there was a relative increase in cell volume with hypertrophy and irregular protrusions; this activation was reversed in both the hippocampus and cortex by the administration of CTS (Fig.6B, E-F). These findings indicated that ligature-induced periodontitis might play an important role in neuroinflammation.

3.6 Effects of ligature-induced periodontitis on interleukins in the cortex

RT-PCR assays were performed to determine the mRNA expression of IL-1β, IL-6, IL-8 and IL-21 in the cortex. In addition, ELISA was used to detect protein levels (Fig. 7). The protein expression of IL-1β, IL-6, IL-8 and IL-21 in the cortex was significantly higher in the CP group than in the control group. In comparison to the CP group, the CP plus CTS group showed a significant reduction in IL-6 and IL-21. High

gene and protein expression of inflammatory factors in the cortex was stimulated by ligature-induced periodontitis and was significantly prevented by CTS.

3.7 Effects of ligature-induced periodontitis on the STAT3 signalling pathway

As shown in Figure 8, Western blot analysis was performed to study the underlying mechanism of neuroinflammation induced by periodontal infection. We detected elevated expression of JAK2, STAT3, and pSTAT3 (Tyr705) in the cortex of the CP group, which was reduced by CTS, suggesting that ligature-induced periodontitis can induce neuroinflammation through pSTAT3 pathway cascades.

3.8 Effects of ligature-induced periodontitis on APP processing

As shown in Figure 9, ligature-induced periodontitis modulated APP processing. Increased expression of APP and two homologs, APLP1 and APLP2, especially APLP2, was found in the CP group (Fig. 9A-C). RT-PCR was performed to detect the mRNA levels of APP secretases, including α-, β- and γ-secretases, which were also upregulated, while these changes were reversed by CTS. After periodontal ligature, there was an approximately threefold increase in the mRNA expression of BACE1 and PS2 and a half-fold decrease in ADAM10 mRNA expression in comparison to that in the control group (Fig. 9D-H). The CP plus CTS group showed reduced mRNA expression related to APP processing in comparison to that in the CP group. Increased expression of the Aβ ratio was detected in the CP group compared to the control group, while these changes were reversed by CTS. According to ELISA, the total Aβ was upregulated and the Aβ ratio (Aβ1-40/Aβ1-42) was nearly doubled in both the plasma and cortex (Fig. 9I-L). The CP plus CTS group showed downregulated production of Aβ in comparison to that in the CP group.

Discussion

Ligature-induced periodontitis in rats induced systemic inflammation and further led to a decline in cognitive function. To our knowledge, this research is the first to identify that ligature-induced periodontitis can impair cognitive function. High expression of inflammatory cytokines, especially IL-6 and IL-21, was found both in the peripheral blood and cortex. Microglia and astrocytes were significantly activated in both the cortex and hippocampus. APP processing was modulated, and the deposition of Aβ was accelerated. The STAT3 signalling pathway was activated in periodontitis-induced systematic inflammation and neuroinflammation, which further resulted in AD-like pathology.

Nearly all studies have focused on the association between inflammation and AD using an acute infection model [19]. For example, a single systematic administration of a large dose of *P. gingivalis*-LPS can cause cognitive impairment [12]. However, few of these studies established a chronic inflammation model, which is more similar to clinical periodontitis and may be more capable of elucidating the possible pathology.

Periodontal ligature is a recognised and widely used method for establishing an animal model of periodontitis. As a promoting factor of plaque formation, ligature can cause continuous accumulation of

plaques and infiltration of inflammatory cells, resulting in the destruction of periodontal connective tissue and loss of alveolar bone [20-21]. Most studies use this model to investigate the condition of local periodontal tissue and the level of inflammation in the periphery, while no studies have focused on the impact of ligature-induced periodontitis on the central nervous system (CNS) [22-23]. The results of our study showed that the expression of inflammatory cytokines was significantly increased in both the periphery and cortex after periodontal ligature, indicating that ligature-induced periodontitis can induce neuroinflammation and reduce cognitive function.

Dysregulated proinflammatory cytokines may induce inflammatory processes and neuroinflammation by activating the STAT3 signalling pathway. In our study, we demonstrated that ligature-induced periodontitis can upregulate the levels of IL-6 and IL-6R as well as IL-21 and IL-21R. IL-6 is an inflammatory cytokine with an important role in the pathogenesis of periodontitis, and previous studies have shown that circulating IL-6 levels are obviously increased in patients with periodontitis. The canonical IL-6 signalling pathway is initiated by binding to IL-6R and the phosphorylation of STAT3 [24-25]. After it binds to its receptor, IL-6 phosphorylates STAT3, which is essential for dimerization and nuclear translocation [26]. In this study, we hypothesized that neuroinflammation can be elicited in a ligature-induced periodontitis model via the STAT3 signalling pathway. To verify this hypothesis, rats were injected with the STAT3 phosphorylation inhibitor CTS, and the results showed that this treatment abolished STAT3 phosphorylation in periodontitis model rats.

Among STAT protein family members, STAT3 is involved in the pathological process of the inflammatory response in the brain and plays a very important role in neural differentiation, nerve regeneration, synaptic plasticity, and energy homeostasis [27-29]. Neuroinflammation is mainly caused by the activation of microglia and astrocytes and the release of cytokines, chemokines or growth factors, all of which can lead to complicated interactions between different types of cells [30]. This work demonstrated that STAT3 activation in the CNS might induce neurotoxicity since the pSTAT3 inhibitor CTS inhibited inflammation and the activation of microglia/astrocytes.

Additionally, neuroinflammation is generally recognised as a prominent feature of AD and probably plays a substantial role in cognitive dysfunction in AD [30]. IL-6, an upstream target of IL-21, has been showed to stimulate the synthesis of Aβ and enhance neuronal damage [31-32]. Both microglia and astrocytes are in a proinflammatory state in AD. According to Schwab and McGeer [33] and Venneti S. et al. [34], activated microglia, which can be labelled by Iba1, can promote the progression of AD by secreting proinflammatory mediators. Additionally, overactivated astrocytes can give rise to the production of inflammatory cytokines and subsequently result in neuronal death, all of which are known to contribute to Aβ production and accumulation [35-36]. In the present study, microglia and astrocytes were both stimulated from a resting state to an activated state, which may have had toxic effects. Meanwhile, Aβ clearance was hindered [37-39].

Continuous chronic peripheral/intrinsic inflammation, such as periodontitis and its chain reactions, is a key feature of AD inflammatory pathology and can also be attributed to Aβ deposition [40]. Previous

study has only demonstrated that systemic inflammation caused by the main pathogenic factor of Gram-negative anaerobic bacteria can lead to learning and memory impairment [41]. AD is characterized by the accumulation of intracellular neurofibrillary tangles and the extracellular deposits of fibrillary Aβ. As a rate-limiting enzyme that initiates Aβ formation, BACE1 plays an essential role in amyloidogenesis [42]. The Aβ ratio (Aβ1-40/Aβ1-42), a more stable biomarker of AD than the concentration of either Aβ1-40 or Aβ1-42, was found to be nearly doubled in both the plasma and cortex [43]. Ligature-induced periodontitis modulated APP processing through the enhanced activity of β- and γ-secretases and increased the production of Aβ, which is also a strong activator of microglia in AD [44]. STAT3 can upregulate β-secretase (BACE1), which affects astrocyte metabolism and increases Aβ production. It also participates in the regulation of neuronal toxicity and the pathological process of AD. After the Stat3 gene is knocked out of astrocytes, plaque deposition and dystrophic axons are reduced, while spatial learning and memory are restored [45-46]. The STAT3 signalling pathway, which is considered to be an important potential target for AD treatment, may be prevalent in brain diseases, and multiple targets participate in the pathological process of AD [47].

Consequently, we speculated that ligature-induced periodontitis can impair cognitive function, induce systematic inflammation and further neuroinflammation, which is represented by stimulation of microglia and astrocytes, modulate APP processing and increase the production of Aβ via the activation of STAT3 cascades. Our study shows that both periodontal bacterial systematic infection and chronic and persistent ligature-induced periodontitis play an important role in the induction and amplification of neuroinflammation in AD.

Conclusions

In summary, we found that ligature-induced periodontitis could increase the inflammation load both in the periphery and the CNS, eventually led to cognitive dysfunction. In this process, STAT3 signaling pathway played an important role in promoting neuroinflammation and modulation of APP processing. Thus, the activation of STAT3 cascades could be involved in the association between periodontitis and AD.

Declarations

Ethics statement: All experimental protocols were approved by the ethical committee of the Animal Care and Experimental Committee of Shanghai Jiao Tong University School of Medicine. The animal experiments were approved by the Animal Care and Welfare Committee of Shanghai Jiao Tong University School of Medicine (approval ID: A-2016-032).

Consent for publication: Not applicable.

Availability of data and materials: The datasets and materials supporting the conclusions of this article are included within the article.

Authors' contributions: YH, XZ, JZ, HL, XX, CQ and ZH: Data curation, Methodology, Validation, Resources. YH, XZ, YL and HC: Formal analysis, Writing-Original Draft, Visualization. WZ and ZS: Writing-Review & Editing, Supervisions, Project administration, Funding acquisition, Conceptualization. All authors read and approved the final manuscript.

Competing interests: None.

Abbreviations

ANOVA: Analysis of variance; BCA: Bicinchoninic acid; BV/TV: Bone volume /Total volume; BS/BV: Bone surface/Bone volume; BMD: Bone mineral density; ELISA: Enzyme-linked immunosorbent assay; GFAP: Glial fibrillary acidic protein; IL: interleukin; PBS: Phosphate-buffered saline; PMSF: Phenylmethylsulfonyl fluoride; PVDF: Polyvinylidene difluoride; RIPA: Radioimmunoprecipitation assay; SDS: Sodium dodecyl sulfate; SEM: Standard error of the mean; STAT: signal transducer and activator of transcription; WB: Western blot.

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Table

Table 1. The sequences of genes and primer pairs.

Target Gene	
GAPDH	Forward:5’-ACAGTCCATGCCATCACTGCC-3’
	Reverse:5’-GCCTGCTTCACCACCTTCTTG-3’
IL-1β	Forward:5’-AACCTGCTGGTGTGTGACGTTC-3’
	Reverse:5’-CAGCACGAGGCTTTTTTGTTGT-3’
IL-6	Forward:5’-GCCCTTCAGGAACAGCTATGA-3’
	Reverse:5’-TGTCAACAACATCAGTCCCAAGA-3’
IL-8	Forward:5’-CATTAATATTTAACGATGTGGATGCG-3’
	Reverse:5’-GCCTACCATCTTTAAACTGCACAAT-3’
IL-21	Forward:5’-GCTCCACAAGATGTAAAGGG-3’
	Reverse:5’-GTGCCTCTGTTTATTTCCTG-3’
APP	Forward:5’-AGAGGTCTACCCTGAACTGC-3’
	Reverse:5’-ATCGCTTACAAACTCACCAACT-3’
APLP1	Forward:5’-TCAGGTCTGCTGATCATGGGAGC-3’
	Reverse:5’-TGGGTGGGGAAGAGGACTTTATTG-3’
APLP2	Forward:5’-CAGAGCGACAGACCCTCATTC-3’
	Reverse:5’-TCTACTCGGGCCAAATGGGT-3’
ADAM10	Forward:5’-GCCTATGTCTTCACGGACCG-3’
	Reverse:5’-TGCCAGACCAAGAACACCATC-3’
ADAM17	Forward:5’-CAGGACGTAATTGAGCGGTTTT-3’
	Reverse:5’-ACGATGTTGTCTGCCAGAAACTT-3’
BACE1	Forward:5’-CGGGAGTGGTATTATGAAGTG-3’
	Reverse:5’-AGGATGGTGATGCGGAAG-3’
PS1	Forward:5’-GAGGAAGACGAAGAGCTGACAT-3’
	Reverse:5’-GAAGCTGACTGACTTGATGGTG-3’
PS2	Forward:5’-GAGCAGAGCCAAATCAAAGG-3’
	Reverse:5’-GGGAGAAAGAACAGCTCGTG-3’

Figures

Figure 1

Effects of periodontal ligature on alveolar bone and periodontal inflammation responses. (A) Images captured by micro-CT scanning of molar region. Bone density analysis of alveolar bone including (B) BV/TV, **p<0.01 compared to the control group, (C) BS/BV, *p<0.05 compared to the control group, (D) BMD, *p<0.05 compared to the control group and #p<0.05 compared to the CP group (n=4-6 per group).

After decalcification, hematoxylin-eosin (HE) staining assay was used to detect the inflammation response in maxillae of rats (E). Microscope images were at a magnification of 100× (bar=200μm).

Figure 2

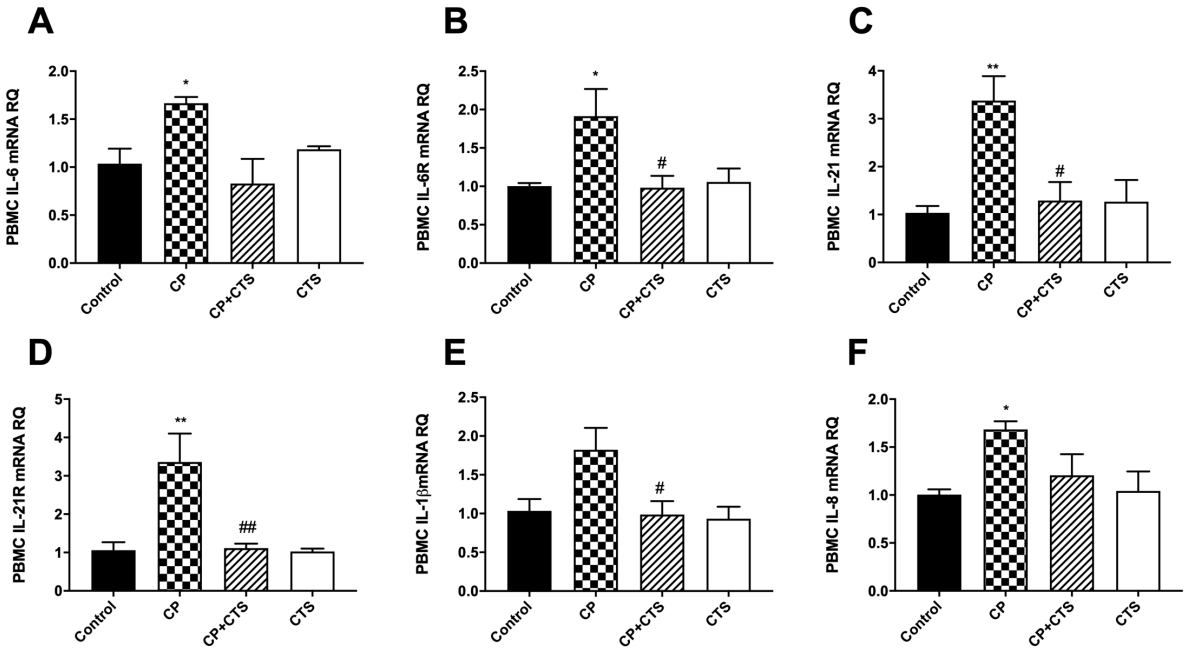

Figure 2

Effects of ligature-induced periodontitis on mRNA level of interleukins in PBMCs. Periodontal ligature induced high expression of IL-1β, IL-8, IL-6, IL-21 and their corresponding receptors (IL-6R and IL-21R) in PBMCs, while these changes were reversed by CTS. The mRNA expression of cytokines in each group: (A) IL-6, (B) IL-6R, (C) IL-21, (D) IL-21R, (E) IL-1β, (F) IL-8. (n=4–8 per group, one-way ANOVA, *p<0.05 and **p<0.01compared to the Control group, #p<0.05 and ##p<0.01 compared to the CP group)

Figure 3

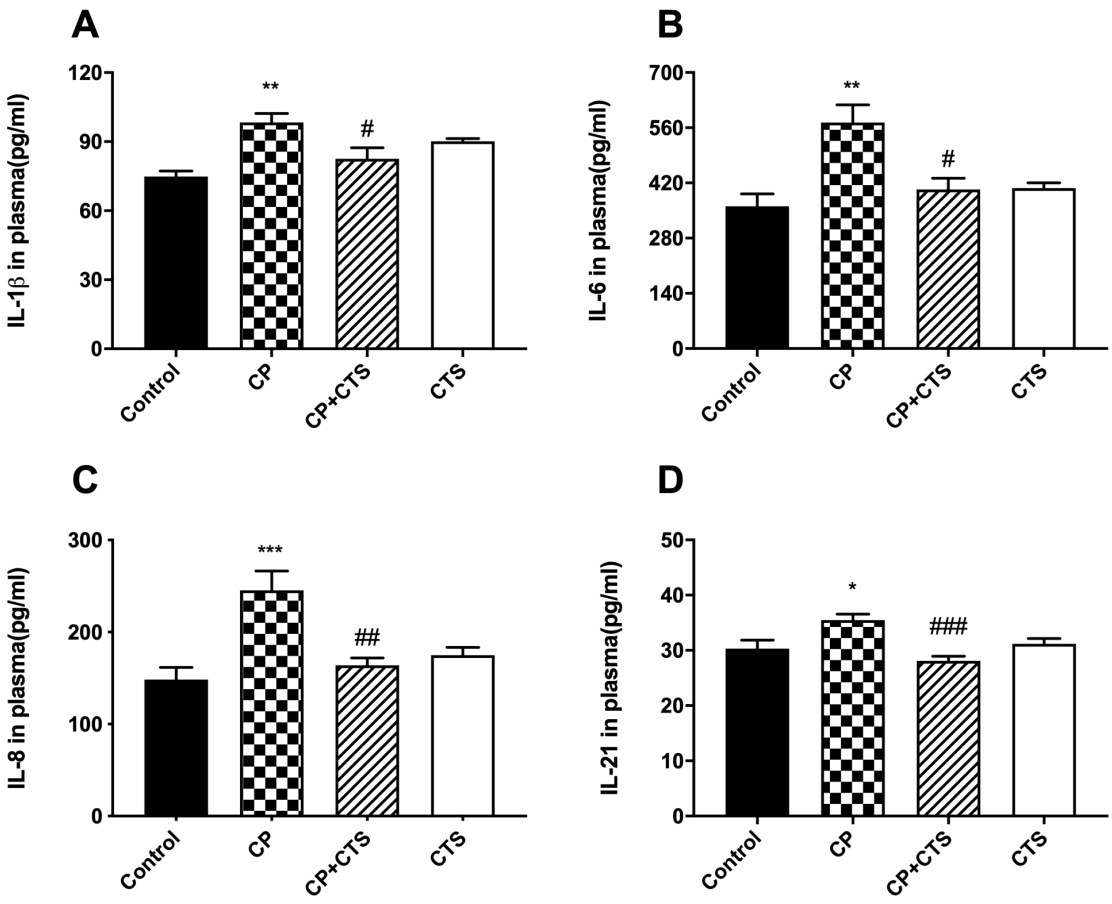

Figure 3

Effects of ligature-induced periodontitis on protein level of interleukins in plasma. ELISA was performed to detect protein levels of interleukins in plasma. Ligature-induced periodontitis induced high expression of inflammatory cytokines (IL-1β, IL-6, IL-8, IL-21) on protein in comparison to the control group, while these changes were reversed by CTS. The protein expression of cytokines in plasma in each group: (A) IL-1β, (B) IL-6, (C) IL-8, (D) IL-21. (n=4–8 per group, one-way ANOVA, *p<0.05, ***p<0.001 compared to the Control group, #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001compared to the CP group)

Figure 4

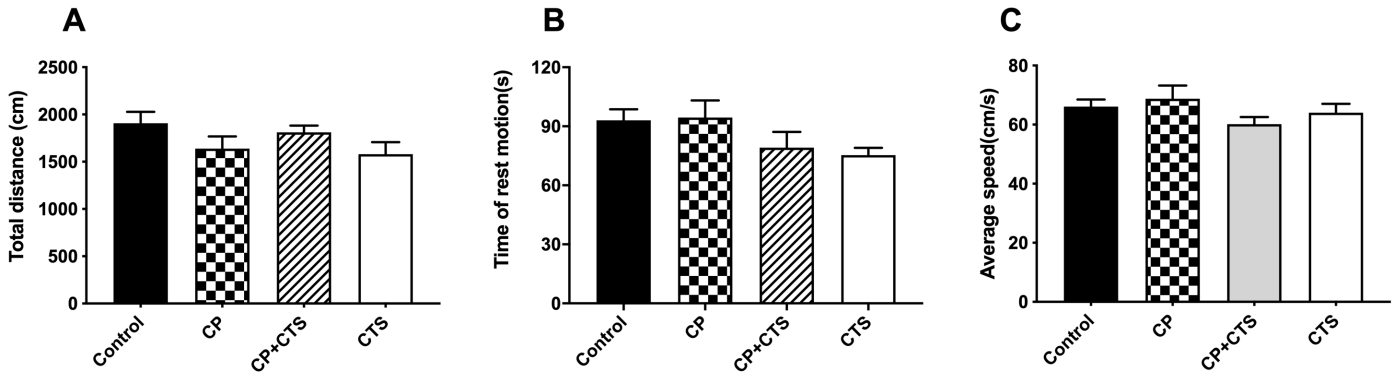

Figure 4

Effects of ligature-induced periodontitis on animal locomotor activity. The open field test (OFT) was used to evaluate the locomotor activities of rats three days after the final administration. (A) Total distance, (B) Time of rest motion, (C) Average speed. Overall, no significant differences were observed between groups in OFT. (n=4-8 per group, one-way ANOVA, p>0.05)

Figure 5

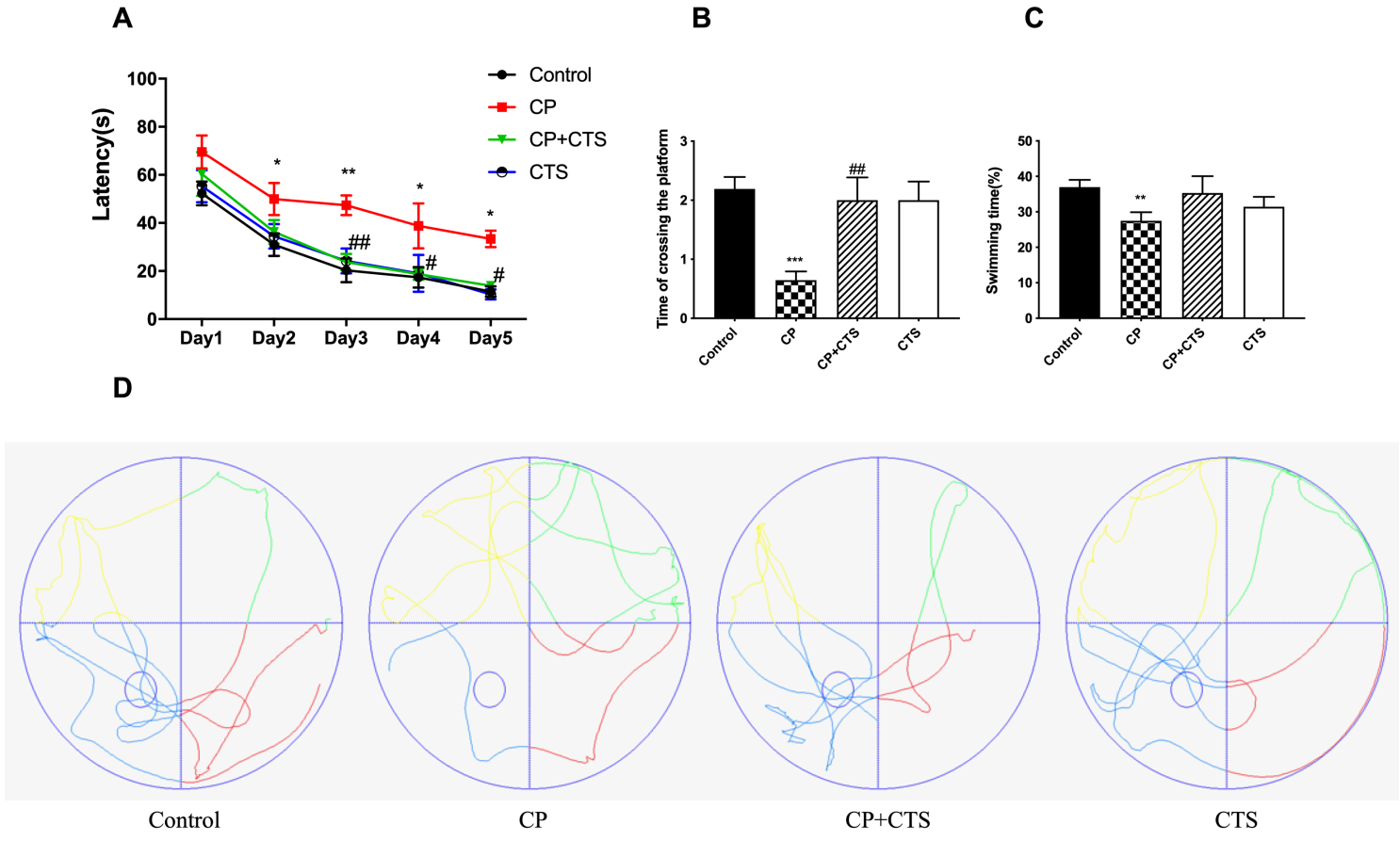

Figure 5

Effects of ligature-induced periodontitis on learning and memory. The Morris Water Maze test (MWM) was conducted to assess the learning and memory ability, including (A) Latency to find the platform during the acquisition phase of the MWM test, (B) Number of platform crossings in the target quadrant, (C)Percentage of time spent in the target quadrant and (D)The typical trajectories of the CP group approximated the arc of a circle, without any crossings over the original platform. The platform is represented by an open circle. (n=4–8 per group, two-way ANOVA, *p<0.05, **p<0.01 compared to the Control group, #p<0.05, ##p<0.01 and ###p<0.001 compared to the CP group)

Figure 6

Effects of ligature-induced periodontitis on microglia and astrocytes in the hippocampus and cortex. Histopathological analysis of brain sections was performed using immunohistochemistry. Microglia were visualized with ionized calcium-binding adaptor molecule 1 (Iba1) and astrocytes were labelled by glial fibrillary acidic protein (GFAP) (arrows). Quantification of Iba1 and GFAP levels in the hippocampus and cortex are shown (C-F). Activated microglia and astrocytes were attenuated by pre-treatment of CTS (200×, bar=200μm). (n=4–6 per group, one-way ANOVA, **p<0.01 and ***p<0.001 compared to the Control group, #p<0.05 and ##p<0.01 compared to the CP group)

Figure 7

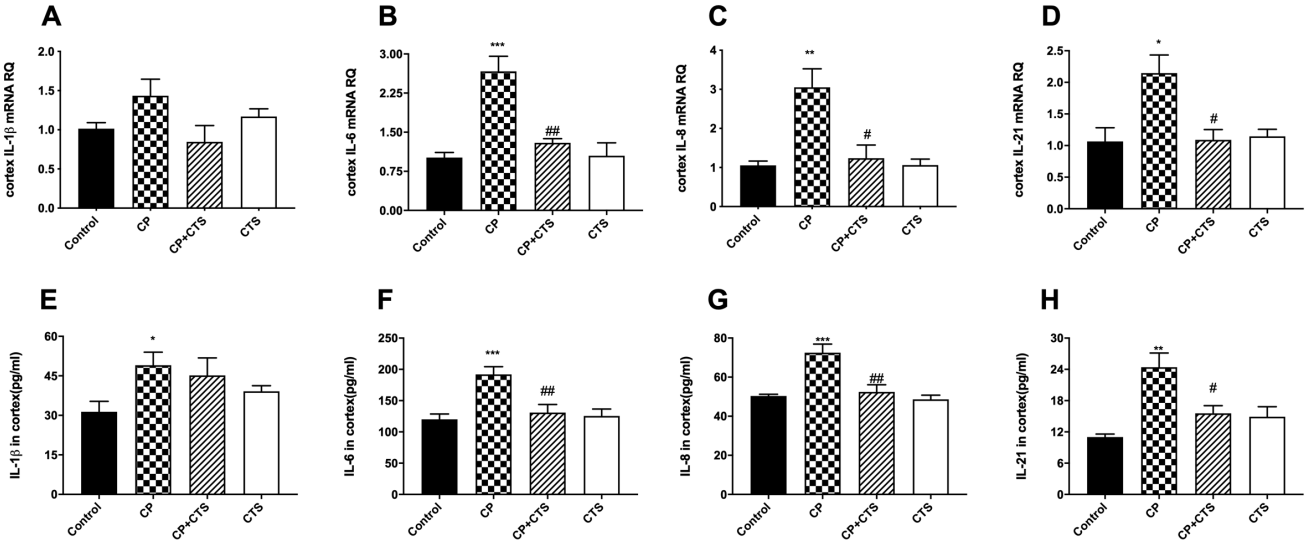

Figure 7

Effects of ligature-induced periodontitis on interleukins in cortex. RT-PCR and ELISA were performed to detect mRNA and protein levels of interleukins in cortex. Periodontal ligature induced high expression of inflammatory factors on both mRNA and proteins in comparison to the control group, while these changes were prevented by CTS. The mRNA level of cytokines in plasma in each group: (A) IL-1β, (B) IL-6,

(C) IL-8, (D) IL-21. The protein level of cytokines in cortex in each group: (E) IL-1β, (F) IL-6, (G) IL-8, (H) IL-21. (control group (Control), periodontal ligature group (CP), periodontal ligature plus CTS group (CP+CTS) and CTS group (CTS); n=4–8 per group, one-way ANOVA, *p<0.05, **p<0.01 and ***p<0.001compared to the Control group, #p<0.05 and ##p<0.01 compared to the CP group)

Figure 8

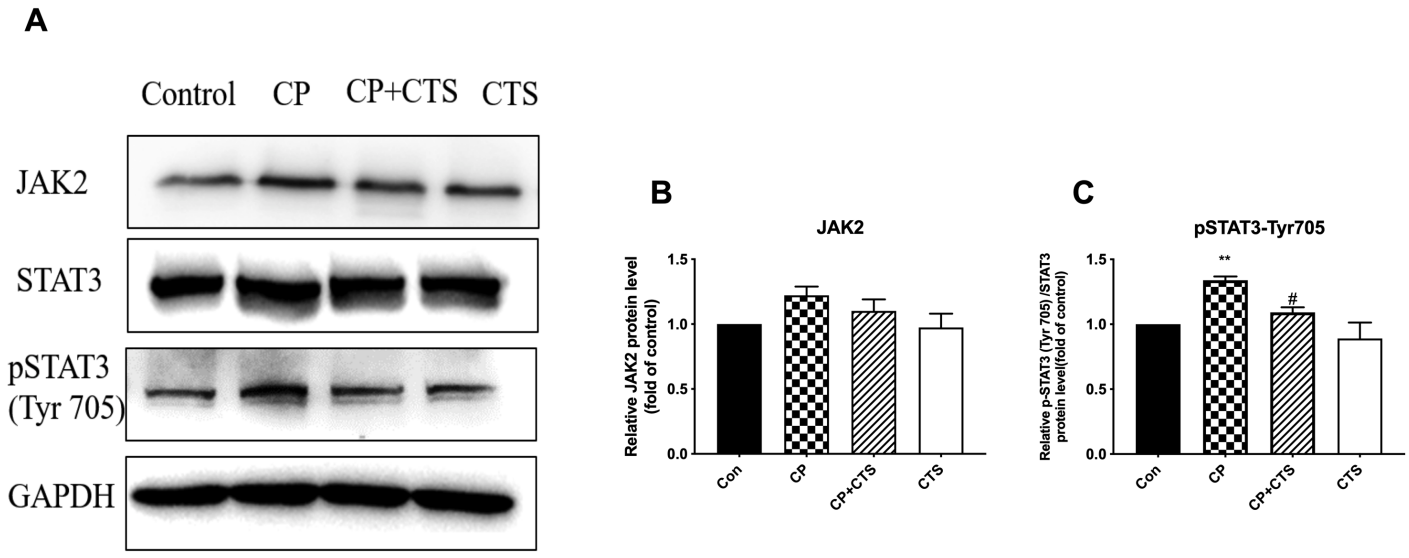

Figure 8

Effects of ligature-induced periodontitis on STAT3 signaling pathway. Expression of JAK2, STAT3 and pSTAT3 (Tyr-705) was further measured by western blot analysis. High expression of pSTAT3 was effectively inhibited by CTS. pSTAT3/STAT3 was upregulated by periodontal ligature. The quantification of related protein expression in each group: (A) JAK2, (B) STAT3, (C) pSTAT3 (Tyr-705)/STAT3. (n=4 per group, one-way ANOVA, *p<0.05 and **p<0.01 compared to the Control group, #p<0.05 and ##p<0.01 compared to the CP group)

Figure 9

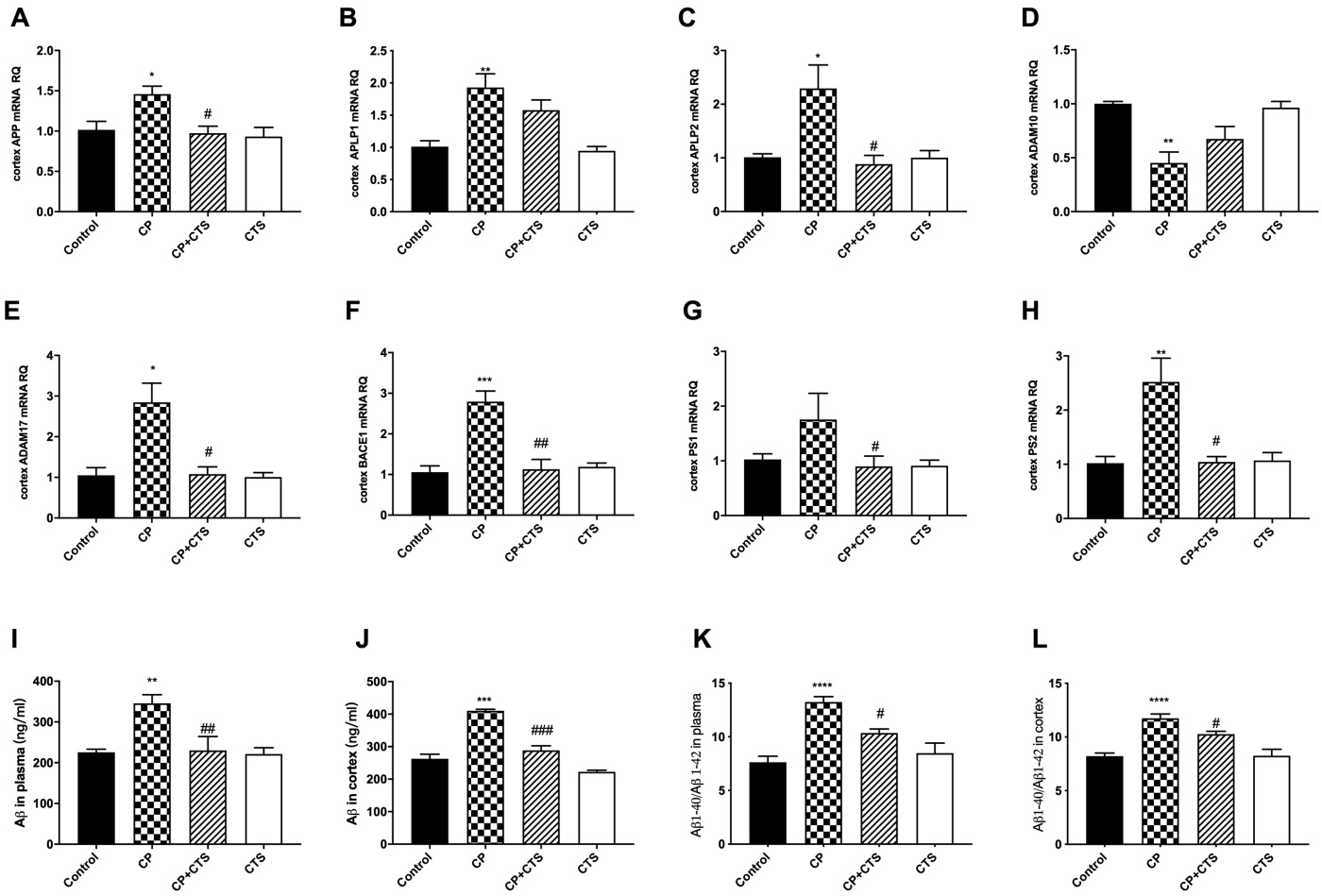

Figure 9

Effects of ligature-induced periodontitis on APP processing. RT-PCR was performed to detect mRNA expression of APP, APLP1 and APLP2 on genes in comparison to the control group, while these changes were reversed by CTS. The mRNA level of APP and the homologs in cortex in each group: (A) APP, (B) APLP1, (C) APLP2. The mRNA level of secretases in cortex in each group: (D) ADAM10, (E)ADAM17, (F)BACE1, (G)PS1, (H)PS2. ELISA was performed to detect the Aβ ratio (Aβ1-40/Aβ1-42) in plasma and cortex: (I) Total Aβ in plasma, (J) Total Aβ in cortex, (K) The Aβ ratio in plasma, (L) The Aβ ratio in cortex. (n=4-8 per group, one-way ANOVA, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared to the Control group and #p<0.05, ##p<0.01 and ###p<0.001 compared to the CP group)

Supplementary Files

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