Ameliorating effects of Juzentaihoto on restraint stress and *P. gingivalis*-induced alveolar bone loss

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Four figures and two tables
Running title: Ameliorating effects of Juzentaihoto on restraint stress
Summary of key finding: We found ameliorating effects of Juzentaihoto on restraint stress- and *P. gingivalis*-induced alveolar bone loss in rats.

ABSTRACT

Objective: Juzentaihoto (JTX) is a traditional Japanese medicine that consists of 10 herbs. The purpose of this study was to evaluate the efficacy of multi-herbal medicine JTX as a preventive and therapeutic drug for periodontal bone resorption and for reducing restraint stress.

Material and methods: *Porphyromonas gingivalis* ATCC 33277 was used for testing the antibacterial activity of JTX and a rat experimental periodontitis model. To evaluate the effect of JTX against *P. gingivalis* infection, we determined the differences in alveolar bone loss among experimental groups. The concentrations of adrenocorticotropic hormones were measured as stress markers, and atrophy of the thymus and spleen was assessed.

Results: JTX had antibacterial activity against *P. gingivalis* ATCC 33277. JTX treatment of mouse bone marrow cells at a concentration of 0.1 μg/ml significantly inhibited osteoclast formation. Administration of JTX to rats with *P. gingivalis* infection and restraint stress significantly reduced alveolar bone loss compared with the case with just the combination of *P. gingivalis* infection and restraint stress. In the restrained groups, stress markers were elevated, and the thymus and spleen were atrophied. The groups with administration of JTX showed not only inhibition of the decrease of weight but

also normalization of corticosterone and cortisol values.

Conclusion: JTX effectively inhibited restraint stress and osteoclastogenesis. It appears that the effects of JTX inhibit the destruction of periodontal tissue by suppressing stress. Our study demonstrated that JTX affects the correlation between restraint stress and periodontitis.

KEY WORDS

Juzentaihoto; *Porphyromonas gingivalis*; alveolar bone loss; stress

1. INTRODUCTION

Dental plaque is a biofilm consisting of multiple species of oral bacteria and their metabolic products. Periodontitis is a chronic inflammatory disease of the supporting tissues of the teeth associated with the emergence of a consortium of Gram-negative bacteria in subgingival plaque.[1] *P. gingivalis* has received considerable attention because it can represent up to 50% of anaerobically cultivable bacteria from adult periodontal lesions.[2] An increase in the severity of periodontitis was also associated with an increase in the subgingival level of *P. gingivalis*, and resolution of the disease was associated with a reduction in the level of the bacterium.[3,4] Furthermore, subgingival implantation of *P. gingivalis* in mice, rats and non-human primates reportedly induced periodontal bone loss.[5,6] These studies in humans and animals implicated *P. gingivalis* as a major causative agent in the development of periodontitis.

P. gingivalis possesses a number of virulence factors that enable it to colonize the oral cavity and to destroy gingival tissues, such as fimbriae, strong proteolytic activity, lipopolysaccharides, vesicles and outer membrane proteins.[7] Besides these direct host–pathogen interactions, periodontitis is exacerbated by risk factors including age, male gender, smoking and diabetes mellitus.[8]

Psychological factors have also been suspected of increasing the risk of periodontitis.[9]

The stress response is thought to be a mediating mechanism between unfavorable psychological conditions and inflammatory periodontal disease. Some studies have reported a higher prevalence of chronic destructive periodontal disease in individuals with psychological stress, which may be associated with acute necrotizing periodontal disease.[10-15] Concerning the mechanism linking stressful conditions and periodontal diseases, there are many reports describing that psychological stress can downregulate the cellular immune response. [16-20]

Kampo medicine is traditional Japanese herbal medicine that also uses substances that are of pharmaceutical grade; it is integrated into the national medical system. The principles of Kampo are based on old Chinese traditional medicines and about one hundred and fifty kinds of compositions of natural herbs and other natural products. Juzentaihoto (JTX; *Shi-Quan-Da-Bu-Tang* in Chinese) is a Kampo medicine that consists of 10 herbs: *Ginseng radix, Astragali radix, Angelicae radix, Rehmanniae radix, Atractylodis lanceae rhizoma, Cinnamomi cortex, Poria, Paeoniae radix, Ligustici rhizoma and Glycyrrhizae radix.* It has been traditionally used for centuries for general malaise. Currently, JTX is widely prescribed for anemia, rheumatoid arthritis, chronic fatigue syndrome and inflammatory bowel diseases. It is also widely used for the prevention of cancer metastasis and infection in immunocompromised patients.[21]

There are various methods of treating periodontal conditions, such as using tetracycline antibiotics and anti-inflammatory agents. Ideal antibacterial substances must be effective against numerous microorganisms, act rapidly, maintain activity at low concentrations, have no side effects and be usable without causing discomfort. Frequently used antibacterial chemicals include povidone iodine, chlorhexidine and cetylpyridinium chloride; in addition, natural antibacterial substances have attracted attention. [22-24]

In the medical field, combinations of Japanese traditional herbal medicine and Western medicine are applied. However, Japanese traditional medicine has rarely been applied in dentistry. In the present study, we investigate the antibacterial effect of JTX and the inhibitory effect of JTX on the differentiation of mouse bone marrow cells into osteoclasts. Furthermore, we used a rat periodontitis model to determine the inhibitory efficacy of JTX on the combination of restraint stress and *P. gingivalis*-induced alveolar bone loss.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The bacterial strain used in this study was *P. gingivalis* American Type Culture Collection (ATCC) 33277. It was grown at 37ºC for 18 h in an anaerobic chamber with an atmosphere of 85% N_2, 10% H_2 and 5% CO_2 in brain heart infusion broth (BHI broth; Becton Dickinson and Company, Sparks, MD, USA) supplemented with 5 mg/ml yeast extract, 5 μg/ml hemin and 0.2 μg/ml menadione.

2.2. Preparation of herbal medicine

Juzentaihoto (JTX) used in this study was obtained from Tsumura & Co., Ltd. (Tokyo). JTX powder was dissolved in phosphate-buffered saline (PBS; pH 7.4) and serially diluted (100 mg/ml, 10 mg/ml and 1 mg/ml) with PBS for use in evaluating the antibacterial activity against *P. gingivalis*. Ten mg/ml JTX was prepared in distilled water for evaluating the inhibitory efficacy of the Kampo medicine on experimental periodontitis in rats. To examine cell viability and osteoclast differentiation, JTX was prepared in α-minimum essential medium (α-MEM; GIBCO, Grand Island, NY, USA) and serially diluted from 100 μg/ml to 0.01 μg/ml.

2.3. Antibacterial activity against *P. gingivalis*

Exponentially growing *P. gingivalis* cells were washed and suspended in PBS to an

optical density of 1.0 at 600 nm. Ten μl of the bacterial suspension was exposed for 5, 15 and 60 minutes to 1 ml of the 100 mg/ml, 10 mg/ml and 1 mg/ml JTX, or the same volume of PBS as a control. At the end of the incubation period, 10-fold serial dilution was carried out in PBS and 100 μl of each dilution was spread onto a BHI blood agar plate. The number of CFU (colony forming units) was determined after 7 days of incubation in an anaerobic atmosphere. Each experiment was performed three times and the mean values of the experiments are shown.

2.4. Cell viability assay

A cell viability assay was performed to examine the effect of JTX on the cytotoxicity of MC3T3-G2/PA6 cells (RIKEN Cell Bank, Tsukuba, Japan), pre-adipocytes similar to bone marrow-derived stromal cells, using a CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay kit, which consisted of a novel tetrazolium compound (MTS) and an electron-coupling reagent (Promega Co., Madison, WI, USA). MC3T3-G2/PA6 cells were placed in 96-well plates at a concentration of 1 x 10^4 cells/well and were grown in α-MEM containing 20% fetal bovine serum (FBS; SAFC Biosciences, Denver, KS, USA) to sub-confluence. The cells were then treated with 100 μg/ml, 10 μg/ml, 1 μg/ml, 0.1 μg/ml and 0.01 μg/ml JTX for an additional 2 days. Subsequently, 20 μl of

MTS solution was added to each well and the cells were incubated for 4 h at 37°C. After incubation, the optical density of the formazan solution was measured at 490 nm. The results are expressed as the mean ± standard deviation (SD) of the relative percentage of viability compared with that of negative controls without JTX (n = 5).

2.5. Osteoclast differentiation assay

Bone marrow cells (1.5 x 10^5 cells/well), obtained from the tibiae of 5- to 8-week-old BALB/c mice, and MC3T3-G2/PA6 cells (1.5 x 10^4 cells/well) were co-cultured for 7 days in the presence of 10^{-8} M 1α,25-$(OH)_2D_3$ (Calcitriol; Wako Pure Chemical Industries, Osaka, Japan) and 10^{-8} M dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) in α-MEM supplemented with 20% FBS in 48-well plates under a 5% CO_2 atmosphere. To determine the inhibitory effects of JTX on osteoclast formation, JTX was added to the culture at final concentrations of 10 μg/ml, 1 μg/ml, 0.1 μg/ml and 0.01 μg/ml and cultured for 7 days. After 7 days, the co-cultured cells were stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts. TRAP-positive multinucleated cells containing three of more nuclei were counted as osteoclasts. The results are expressed as the means ± SD of triplicate cultures.

2.6. Animals

Forty-eight male, specific-pathogen-free (SPF), 4-week-old, Sprague-Dawley rats, each weighing 50–60 g, were used in the experiments. The rats were obtained from a commercial farm (Nihon SLC, Shizuoka, Japan) and housed in cages throughout the experimental period to facilitate successful isolation. The rats were fed a standardized diet of hard briquettes and water, and were maintained under a 12-h light-dark cycle (lights on 08:00–20:00) at a temperature of 22ºC and relative humidity of 50%.

2.7. Experimental procedure

The body weight of the rats was recorded every 2 days in the morning from the beginning to the end of the experimental period. The rats were given sulfamethoxazole (1 mg/ml) and trimethoprim (200 μg/ml) in drinking water for 4 days to reduce the original oral flora, followed by a 4-day antibiotic-free period before being challenged with *P. gingivalis*. The rats were orally infected with *P. gingivalis* ATCC 33277, which was suspended in 5% carboxymethylcellulose, and each rat received 0.5 ml (1.5×10^{10} cells/ml) by oral gavage three times at 48-h intervals. Restraint stress was induced by enclosing each animal in flexible wire mesh (5 × 5 mm) shaped to fit its body from 20:00 to 8:00 (12 h) for the experimental period. To examine the efficacy of JTX for

periodontal bone resorption and for reducing restraint stress, a 10 mg/ml solution was used for their drinking water. The estimated dose levels in rats were calculated to match the typical estimated human exposure. The six rats in each cage belonged to the same group.

The rats were randomly divided into the following eight groups of six rats each: group A, serving as *P. gingivalis*-uninfected control (sham); group B, administered JTX; group C, infected with *P. gingivalis*; group D, infected with *P. gingivalis* and administered JTX; group E, exposed to restraint stress; group F, exposed to restraint stress and administered JTX; group G, exposed to restraint stress and infected with *P. gingivalis*; and group H, exposed to restraint stress, infected with *P. gingivalis* and administered JTX (Fig. 1). In the unrestrained groups (groups A through D), the rats were denied food and water for the same time period, but were not restrained (Fig. 1).

The experimental procedures of this study were reviewed and approved by the Committee of Ethics on Animal Experiments of Kanagawa Dental University and were carried out under the guidelines for animal experimentation of Kanagawa Dental University, which are in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.8. Measurement of alveolar bone loss

One aspect of periodontal disease is the resorption of alveolar bone. The upper jaws were defleshed after 10 min in an autoclave at 15 pounds/inch^2, and were then immersed in 3% hydrogen peroxide, rinsed, air-dried and stained with 1% methylene blue. Horizontal alveolar bone resorption around the maxillary molars was evaluated morphometrically. The distance between the cemento-enamel junction (CEJ) and the alveolar bone crest (ABC) was measured at seven buccal sites per rat.[25] Measurements were made under a dissecting microscope (x 40) fitted with a digital high-definition system (digital HD microscope VH-7000; Keyence, Osaka, Japan) that was standardized to provide measurements in millimeters.

2.9. Measurement of corticosterone and cortisol

In order to clarify the ameliorative effect of JTX on restraint stress in the rats, blood was taken by cardiac puncture under anesthesia (Veterinary Ketalar® 50, Sankyo Co., Ltd., Tokyo, Japan) to measure the corticosterone and cortisol when the animals were sacrificed. Blood samples were centrifuged, separated into serum and kept frozen at -20ºC. In the case of corticosterone and cortisol, serum was assayed using a commercial radioimmunoassay kit (Immunotech, Beckman Coulter Co., Fullerton, CA, USA).

2.10. Statistical analysis

Differences among experimental groups were analyzed with Fisher's protected least significant difference (PLSD) by one-way analysis of variance. The 5% level of significance was selected for rejecting hypotheses. Computations were performed using a statistical software program (STATVIEW version 5.0; Abacus Concepts, Inc., Berkeley, CA, USA).

3. RESULTS

3.1. Antibacterial activity against *P. gingivalis*

The treatment of *P. gingivalis* with 100 mg/ml, 10 mg/ml and 1 mg/ml JTX reduced the number of viable cells. At concentrations of 100 mg/ml and 10 mg/ml JTX, antibacterial activity against *P. gingivalis* was observed for the 60 min treatment and the numbers of CFU of *P. gingivalis* decreased from $4.12 \pm 0.05 \times 10^7$ to zero per milliliter and $1.93 \pm 0.10 \times 10^4$ per milliliter, respectively (Fig. 2).

3.2. Effect of JTX on cell viability

The MTS assay was performed to assess the effect of JTX on MC3T3-G2/PA6 cell viability and to determine the appropriate concentration for treating cell cultures. Compared with untreated cells, JTX concentrations up to 10 μg/ml showed no significant effect on cell viability. Therefore, JTX was used at concentrations of 10 μg/ml, 1 μg/ml, 0.1 μg/ml and 0.01 μg/ml in the osteoclast formation.

3.3. JTX inhibits osteoclast differentiation

TRAP-positive multinucleated osteoclasts were formed in the co-cultures of MC3T3-G2/PA6 cells and mouse bone marrow cells in the presence of 1α,25-$(OH)_2D_3$ and dexamethasone. Treatment with JTX (at concentrations of 10 μg/ml, 1 μg/ml and 0.1 μg/ml) significantly inhibited osteoclast formation ($^{**}p < 0.01$, $^{*}p < 0.05$, Fig. 3). However, no inhibitory effect was observed in co-culture cells with 0.01 μg/ml JTX.

3.4. Alveolar bone loss

To evaluate the effect of JTX against restraint stress- and *P. gingivalis*-induced alveolar bone loss, bone level differences of the rats were determined. Morphometric bone levels were shown in Fig. 4A. The mean [± standard error of the mean (SEM)] CEJ-ABC distance at each of the seven measurement sites was compared among groups (Fig. 4B).

As alveolar bone decreased, the CEJ-ABC distance increased in the eight experimental groups. The bone levels of groups A, B, C and D were 0.19 ± 0.04 mm, 0.20 ± 0.02 mm, 0.37 ± 0.01 mm and 0.24 ± 0.02 mm, respectively. The level of bone loss in group C, which was infected with *P. gingivalis*, was significantly greater than that in group A ($p < 0.05$), indicating that *P. gingivalis* infection was associated with bone loss. The bone loss level of the group administered JTX with *P. gingivalis* infection was remarkably less than that of the *P. gingivalis* infection group ($p < 0.05$), whereas administration of JTX itself had no effect on alveolar bone resorption. The bone loss level of group G with combined restraint stress and *P. gingivalis* infection was 0.43 ± 0.05 mm, which was 2.24-fold higher than that of the control without *P. gingivalis* infection (group A) ($p < 0.01$). Although no significant difference of bone loss level was observed between control group A and stress group E, the combination of restraint stress and *P. gingivalis* infection (group G) exacerbated the alveolar bone resorption in comparison to that with *P. gingivalis* infection alone (group C) ($p < 0.05$). The administration of JTX to cases of *P. gingivalis* infection with restraint stress (group H) significantly reduced the bone resorption ($p < 0.05$). Since the bone level of group H was comparable to that of group D, without restraint stress, administration of JTX may alleviate the exacerbating effect of restraint stress on alveolar bone resorption induced by *P. gingivalis*.

3.5. The weights of body, thymus and spleen

At the beginning of the experimental period, all rats had similar body weights. Because the rats were in their growth period, their body weights increased during the experiment. In the restrained groups (groups E through H), overall lower increases in body weights were observed compared with those in the unrestrained groups (groups A through D). In particular, in restraint stress group E and combined restraint stress and *P. gingivalis* infection group G, the body weights were significantly lower than those in control group A and *P. gingivalis* infection group C ($p < 0.01$ and $p < 0.05$), respectively, at the end of the experimental period (Table 1). Interestingly, the administration of JTX (groups F and H) inhibited the decrease of body weight under the restrained condition and the body weights of the rats were similar to those in control group A. The mean body weight in group B, in which the rats were administered JTX, was higher than that in control group A. The weights of thymus and spleen were measured on day 30. No significant differences were observed in thymus and spleen weights among unrestrained groups (groups A through D). However, thymus and spleen weights were significantly lower in restraint stress groups E and G than in unrestrained groups A and C, respectively ($p < 0.01$ and $p < 0.05$), indicating that the stress caused remarkable

atrophy of the thymus and spleen. On the other hand, the weights of the thymus and spleen in the groups administered JTX under the restraint stress condition (groups F and H) were similar to those in groups B and D, respectively.

3.6. Systemic biomarkers of stress

Blood cortisol and corticosterone levels are shown in Table 2. In the restrained group (group E), corticosterone and cortisol values were significantly higher than those in the control (group A) ($p < 0.05$). On the other hand, the administration of JTX significantly decreased the corticosterone and cortisol levels in group F, which was exposed to restraint stress and administered JTX, compared with those in restraint stress group E ($p < 0.01$). Both hormone levels in group F were comparable to those in control group A.

4. DISCUSSION

Periodontal diseases are infectious and induce inflammation in the supportive tissues of teeth in response to the accumulation of pathogens in the subgingival crevice. The rat experimental periodontitis model used in this study is a relatively simple *in vivo* model in which a single periodontal organism infects the animal, leading to destructive

periodontitis.

Kampo medicines have been suggested to have antibiotic or prebiotic effects.[26] Some constituent herbs of JTX and its ingredients have been reported to have antimicrobial effects.[27,28] Especially, the compounds of *Glycyrrhizae radix* were complete growth inhibition of *P. gingivalis*.[29] Therefore, *Glycyrrhizae radix* may play an important role in the bactericidal effect of JTX against *P. gingivalis*. The antibacterial activity against *P. gingivalis* may be explained by the synergism between the constituents in the herbal composites. In this study, the antibacterial activity against *P. gingivalis* was observed with 100 mg/ml and 10 mg/ml of JTX treatment. The treatment of *P. gingivalis* with JTX reduced the number of viable cells in a time- and concentration-dependent manner. Thus, JTX may exhibit potential combinatory effects of its herbs on *P. gingivalis*-induced alveolar bone loss in rats. JTX may inhibit the adherence and colonization of *P. gingivalis* in rat oral cavity. Moreover, 0.1 μg/ml JTX significantly inhibited osteoclast formation (Fig. 3). *Angelicae gigantis radix* had significantly decreased both TNF-alpha-induced TRAP positive cells and TRAP activity.[30] Furthermore, JTX includes immmunomodulating substances. Glycyrrhizin extracted from *Glycyrrhizae radix* and the extracts of *Astragali radix* have anti-inflammatory.[31,32] In addition, the extracts from *Ginseng radix*, *Cinnamomi cortex*, *Glycyrrhizae radix*,

Astragali radix and Paeoniae radix have antioxidative activities.[33-37]

Alveolar bone resorption was greater in the infection with *P. gingivalis* group (group C) than in the sham group (group A). Administration of JTX along with *P. gingivalis* infection group (group D) significantly reduced alveolar bone loss (Fig. 4).

A number of herbal formulas of traditional Japanese medicines are characterized by the use of mixtures of several herbs in a single formula. It has been postulated that, in multiherbal formulas, the pharmacological activity of one single herb is either potentiated or prolonged, and/or its adverse effects reduced, due to synergistic or antagonistic effects, by the addition of other herbs. A component of JTX suppressed osteoclast differentiation from bone marrow macrophages treated with receptor activator of nuclear factor kappa-B ligand (RANKL) without any cytotoxicity. This component also significantly reduced RANKL-induced expression of transcription factors, c-Fos and nuclear factor of activated T-cells, as well as the osteoclast marker TRAP.[38,39] Since no significant inhibitory effect on cell viability of MC3T3-G2/PA6 cells was observed with JTX, the same suppression of signal transduction may result in a decrease of signal transduction and a decrease of the level of osteoclast formation. One study reported that JTX caused the induction of interleukin (IL)-12 and subsequent activation of natural killer T (NKT) lymphoid cells.[40] Other work has mentioned IL-18

induction as a possible result of JTX. These cells have a role in both innate and adaptive immune responses.[41] Cytokines, such as IL-12 and IL-18, are also important activators of NKT cells.[42] Yamada *et al*.[43] investigated the in vitro effect of IL-18 on osteoclastic bone-resorbing activity, and found that when IL-18 was applied to osteoclast-enriched cell cultures, bone-resorbing activity decreased.

Psychological factors have been suspected of increasing the risk of periodontitis.[9] Genco *et al.*[16] reported that analyses of the role of stress in infectious processes, such as periodontal disease, should consider the overall effects of stressors appraised by the brain. Breivik *et al*.[44] demonstrated that periodontal disease susceptibility and progression could be explained, at least in part, by brain neuroendocrine-immune regulatory mechanisms. Genetically determined hypothalamo-pituitary-adrenal (HPA) axis reactivity also appears to play an important role, and periodontal disease is likely to send feedback signals to the brain.[45]

Thymus and spleen weights in the restrained group decreased 28% and 29% compared with those in the control, respectively. However, the thymus and spleen weights of the group with restraint stress and JTX administration recovered to 103% and 100% of those of the control, respectively. These results indicate the physiological burden caused by restraint stress in rats and that JTX relieved this stress (Table 1). The blood

glucocorticoid levels were measured as markers of stress adaptation. In the restrained group, the levels of the hormone markers cortisol and corticosterone were elevated with involution of the thymus and spleen (Table 2). Our results showed that the administration of JTX could ameliorate the alveolar bone loss induced by restraint stress, possibly by preventing reductions of high glucocorticoid response. As shown in Table 2, corticosterone and cortisol values were decreased by JTX administration.

The combination of restraint stress and *P. gingivalis* infection (Fig. 4, group G) resulted in significantly higher attachment and alveolar bone loss than in *P. gingivalis* infection alone. The administration of JTX along with the combination of restraint stress and *P. gingivalis* infection decreased the alveolar bone resorption (Fig. 4, group H). However, restraint stress alone did not induce alveolar bone loss (Fig. 4, group E).

In the combined restraint stress and *P. gingivalis* infection group, it was suggested that there may be some compensation for the reduction in the lymphatic organ size following stress, and that the immune system of restrained rats was weaker, thereby promoting alveolar bone loss. The present study showed that administration of JTX decreased the induction of stress markers, and resulted in lower attachment and alveolar bone loss than in restraint stress with *P. gingivalis* infection. These results indicate that JTX may prevents the physiological burden caused by restraint stress in rats. With

regard to glucocorticoid regulation, *Ginseng radix* may be involved in its beneficial action. Saponins of *Ginseng radix*, a component of JTX, similarly increased adrenal cAMP, a secondary messenger of adrenocorticotropic hormone, and thereby stimulated the synthesis and secretion of corticosteroids in normal but not hypophysectomized rats.[46] Ginseng saponins also acted as a functional ligand for glucocorticoid receptors.[47] Since glucocorticoids are considered to be essential hormones for overcoming acute physical stress, these ingredients may facilitate the resistance, coping and adaptation responses of organisms to stress through modulation of the glucocorticoid secretion system. These actions may be involved in the anti-stress effect of JTX.

Kato *et al*.[48] reported that the intestinal microflora affects the impact of the multi-herbal product JTX on gene expression in the gut and liver. The administration of JTX changes the intestinal microflora and, as a result, changes the expression levels of HSP105 and HSP70 in the liver and intestine. The expression levels of HSPs in these organs may correspond to the level of inflammation produced in the intestinal tract and JTX plays a role to reduce the inflammation. We showed that JTX has two functions that effectively inhibited restraint stress and osteoclastogenesis in this study. It appears that the effects of JTX inhibit the destruction of periodontal tissue by suppressing stress. JTX appears to inhibit the progression of periodontal disease as well as to reduce stress.

In conclusion, the present results suggest that JTX ameliorates stress and inhibits the progression of periodontal disease by suppressing the maturation of osteoclasts. In addition, these findings suggest that restraint stress may play a role in periodontal disease. The obtained findings may lead to the use of JTX as a therapeutic drug in dental care, such as for periodontitis. However, further research is required to clarify the effective ingredients in JTX and to discover the mechanism underlying its action.

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Conflict of interest

The authors have no conflicts of interest to declare.

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FIGURE LEGENDS

Fig. 1 - Experimental design. The rats were divided into eight groups (n = 6/group): group A, control (not infected with *P. gingivalis*); group B, administered JTX; group C, infected with *P. gingivalis*; group D, infected with *P. gingivalis* and administered JTX; group E, exposed to restraint stress; group F, exposed to restraint stress and then administered JTX; group G, exposed to restraint stress and infected with *P. gingivalis*; and group H, exposed to restraint stress, infected with *P. gingivalis* and administered JTX.

Fig. 2 - Antibacterial effect of JTX on *P. gingivalis*. Bacterial cells were treated with 100 mg/ml (▲), 10 mg/ml (△) or 1 mg/ml (●) JTX or PBS (○) for the indicated period.

Fig. 3 - JTX inhibits osteoclast differentiation of BALB/c mouse bone marrow cells co-cultured with MC3T3-G2/PA6 cells. After incubation for 7 days, co-cultured cells were stained for TRAP (A), and TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts (B). Results are expressed as the mean ± SD of triplicate cultures. $^{**}p < 0.01$, $^{*}p < 0.05$.

Fig. 4 - Morphometric bone levels. The skulls of the rats were defleshed and stained with 1% methylene blue. The skeletal specimens were as follows: group A, control; group B, administered JTX in drinking water; group C, infected with *P. gingivalis*; group D, infected with *P. gingivalis* and administered JTX; group E, exposed to restraint stress; group F, exposed to restraint stress and administered JTX; group G, exposed to restraint stress and infected with *P. gingivalis*; and group H, exposed to restraint stress, infected with *P. gingivalis* and administered JTX (A). Measurement of bone levels was performed by comparing the distance from the cemento–enamel junction (CEJ) to the alveolar bone crest (ABC) at seven palatal sites on three molars on the left side of the maxilla. Values indicate mean bone loss levels ± standard error of the mean (n = 6/group) (B). $^{**}p < 0.01$,$^{*}p < 0.05$.

Table 1- A comparison of the body, thymus, and spleen weight at the end of the experimental period.

Group	Weight (mean ± SD)		
	Body (g)	Thymus (g)	Spleen (g)
A	144.28 ± 7.25	0.35 ± 0.06	0.29 ± 0.04
B	158.28 ± 5.77	0.40 ± 0.04	0.29 ± 0.04
C	150.33 ± 5.75	0.35 ± 0.06	0.28 ± 0.01
D	152.00 ± 7.80	0.36 ± 0.02	0.31 ± 0.02
E	131.50 ± 7.80[a]	0.25 ± 0.02[a]	0.21 ± 0.02[a]
F	145.00 ± 8.18[b]	0.36 ± 0.02[b]	0.29 ± 0.04[b]
G	131.16 ± 8.70[c]	0.27 ± 0.01[c]	0.23 ± 0.02[c]
H	144.83 ± 6.08	0.32 ± 0.02	0.27 ± 0.03

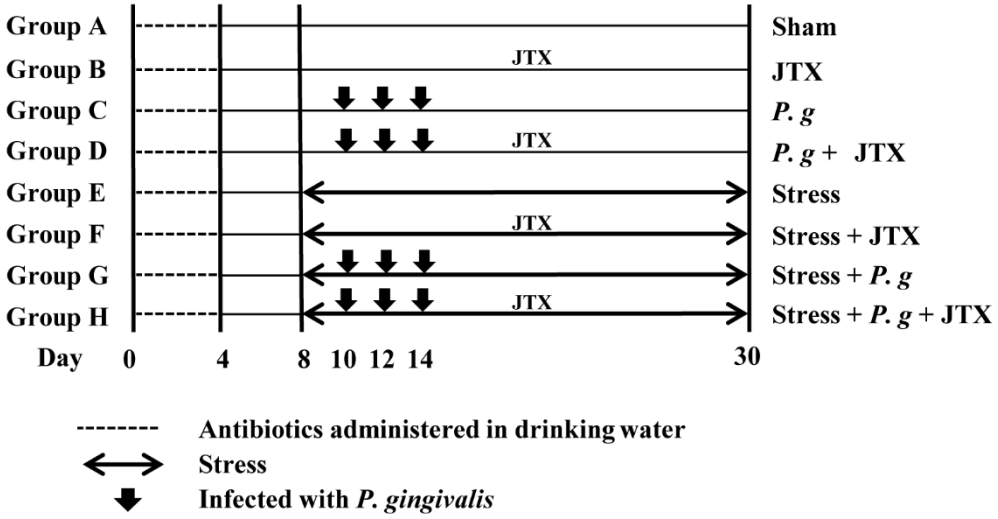
[a]Significantly different ($p < 0.01$) from Group A.
[b]Significantly different ($p < 0.05$) from Group E.
[c]Significantly different ($p < 0.01$) from Group C.

Table 2 - Blood cortisol and corticosterone levels.

Group	Cortisol (μg/dl)	Corticosterone (ng/ml)
A	1.23 ± 0.19	294.16 ± 64.10
E	1.75 ± 0.50[a]	426.00 ± 69.20[a]
F	0.82 ± 0.35[b]	247.83 ± 47.84[b]

[a]Significantly different ($p < 0.05$) from Group A.

[b]Significantly different ($p < 0.01$) from Group E.



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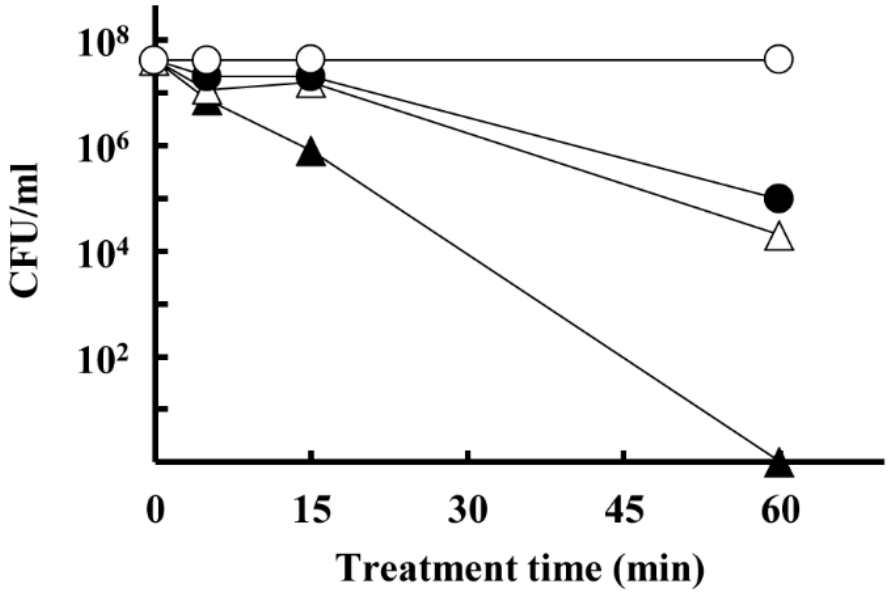

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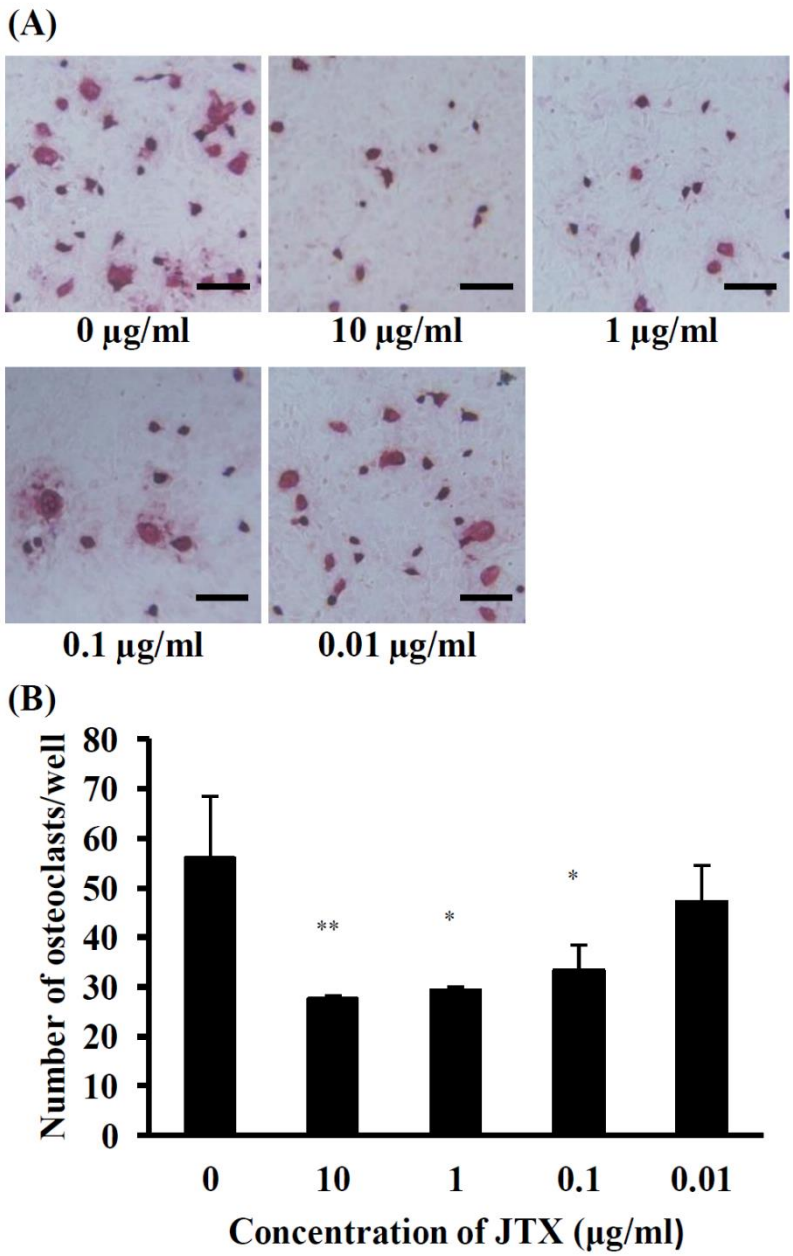

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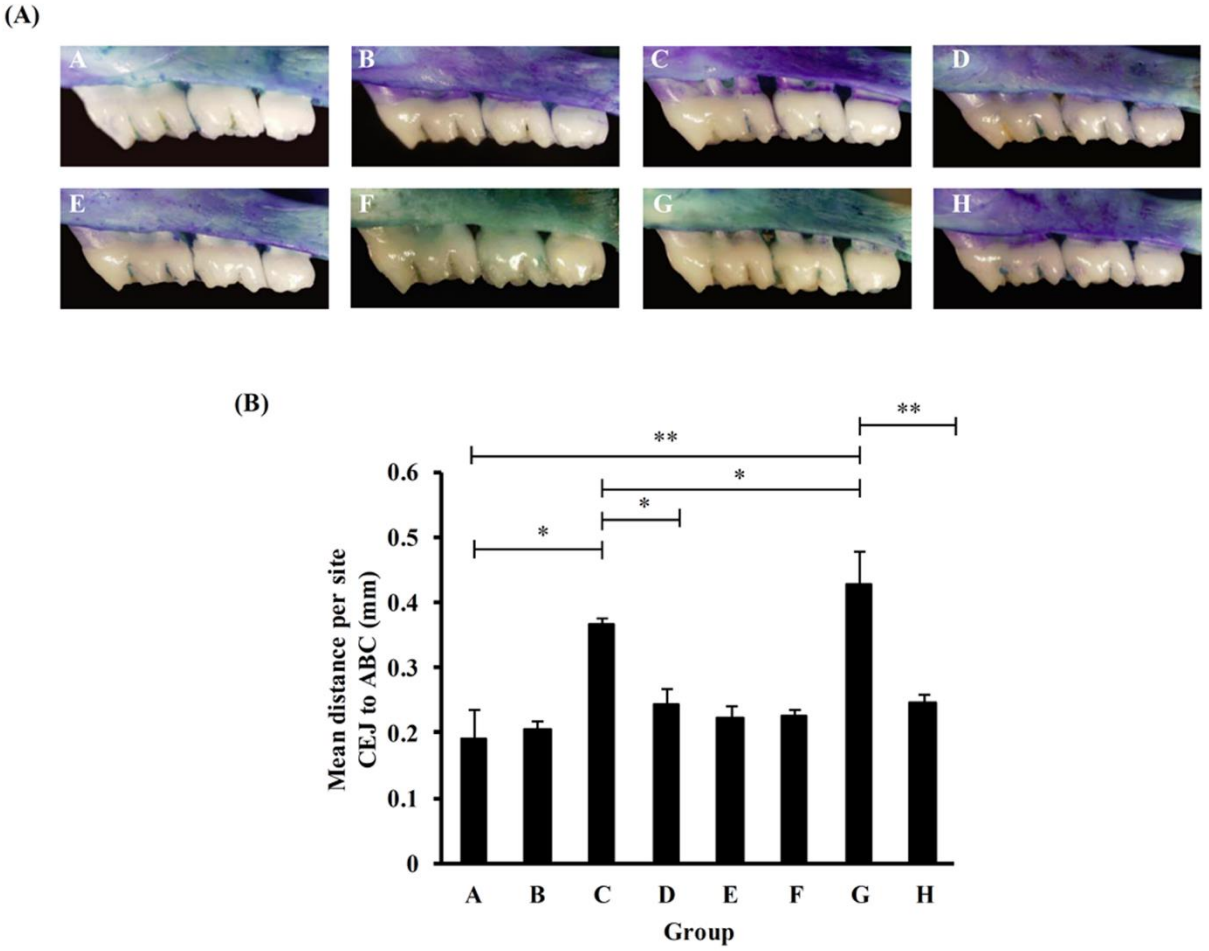

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