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Restraint stress enhances alveolar bone loss in an experimental rat model

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Background and Objective: The purpose of the present study was to examine the effects of restraint stress on periodontal breakdown resulting from *Porphyromonas gingivalis*-challenged periodontitis in rats.

Material and Methods: To examine the influence of restraint stress on periodontal breakdown, rats were orally challenged with the periodontal pathogen *P. gingivalis*. Twenty male, specific pathogen-free (SPF) 3-wk-old, Sprague-Dawley rats were divided into four groups: group A (controls), group B (exposed to restraint stress for 12 h/d for 22 d), group C (orally challenged with *P. gingivalis*), and group D (exposed to restraint stress for 12 h/d for 22 d and orally challenged with *P. gingivalis*). After 22 d, all animals were killed. The distance from the alveolar bone crest to the cemento–enamel junction was determined, concentrations of adrenocorticotropic hormone were measured as stress markers, and atrophy of the thymus and spleen were assessed. In addition, the furcation area of the maxillary molars was examined histologically, while gingival cytokine gene expression was assessed by mRNA using reverse transcription–polymerase chain reaction (RT–PCR).

Results: In the restrained group, all stress markers were elevated, and the thymus and spleen were atrophied. Combined restraint stress and oral challenge with *P. gingivalis* resulted in significantly higher bone loss, and osteoclasts were observed. RT–PCR analysis revealed low cytokine gene expression in the restrained groups.

Conclusion: These results suggest that the presence of restraint stress significantly enhances the progression of *P. gingivalis*-challenged periodontitis in rats.

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Periodontal diseases are infections associated with pathogenic bacteria that colonize the subgingival area, causing inflammation, which may lead to the destruction of periodontal tissues. Periodontal diseases in the past have been characterized as a broad group of infections with multiple bacterial etiologies. However, recent evidence strongly suggests that the onset of adult progressive periodontitis is triggered by a primary pathogen such as *Porphyromonas gingivalis*. This organism possesses a variety of virulence factors, including fimbriae, lectin-like adhesins, capsular polysaccharide, lipopolysaccharide, hemagglutinins and hemolysins, as well as numerous proteolytic enzymes (1). Contribution of *P. gingivalis* to alveolar bone loss seems to be supported by the stimulation of osteoclasts, which induces bone destruction and inhibits bone formation (2). Besides these direct host–pathogen interactions, periodontitis is exacerbated by risk factors represented by age, male gender, smoking and diabetes mellitus (3). Psychological factors have also been suspected of increasing the risk of periodontitis (4). Epidemiological studies, searching for connections between different psychological conditions and progression of

periodontitis, have been published (5). The stress response is thought to be a mediating mechanism between unfavorable psychological conditions and inflammatory periodontal disease. Several mechanisms are suspected of linking the stress response to periodontal disease: increased nicotine consumption; less effective oral hygiene behavior; changed nutritional habits; reduced saliva flow; effects of stress on gingival circulation; and stress-associated alterations in the immune response (4). Furthermore, stress may also act as a risk factor for periodontal disease through catecholamine-induced bacterial growth (6,7). Emotional stress is one of the subject-based factors found to have an effect on immune response and susceptibility to infection. The term 'stress' defines the psychophysiological reactions of the body to a variety of emotional or physical stimuli that threaten homeostasis, and these reactions have been found to exacerbate the outcome of bacterial and viral infectious diseases in human and animal models (8,9). The effects of stress on infectious diseases are thought to be mediated by products of the nervous and the neuroendocrine systems that are released under stress conditions and modulate the function of neutrophils, lymphocytes and macrophages, thereby affecting the outcome of infectious disease (10–13). The expression of interferon-γ (IFN-γ) receptors has been found to be reduced in macrophages from stressed animals (10). The effects of emotional stress on periodontal diseases have been studied in human and animal models. Some human studies have found a positive correlation between negative life events and indices of periodontal disease, thus suggesting that psychological factors involved in stress may increase the susceptibility to periodontitis (14,15). In a large epidemiological study, Genco *et al*. (16) showed that the inability to cope with stress is directly correlated with more severe periodontal destruction. To date, however, the number and scope of human studies have been limited. One early animal study has shown that restraint stress increased bone loss in rats and hamsters (17). Breivik *et al*. (18), in a series of studies with a rat model of ligature-induced alveolar bone loss, showed that the central nervous system, in general, and stress, in particular, could accelerate alveolar bone loss. However, little is known about the cellular functions that are modulated by restraint stress during periodontal infection. Therefore, we investigated the effects of restraint stress and periodontitis, both alone and in combination, on the outcome of experimental periodontitis in rats.

Material and methods

Bacterial strains and growth conditions

The bacterial strain used was *P. gingivalis* ATCC 33277. *P. gingivalis* strains were grown at 37°C for 18 h, in brain heart infusion broth (Difco, Detroit, MI, USA) supplemented with 5 mg/ml yeast extract, 5 μg/ml hemin and 0.2 μg/ml menadione, in an anaerobic chamber with an atmosphere of 85% N_2, 10% H_2 and 5% CO_2.

Animals and experimental periodontitis

Twenty male, specific pathogen-free (SPF), 3-wk-old, Sprague-Dawley rats, each weighing 40–60 g, were used in the experiments and were obtained from a commercial farm (Nihon SLC, Shizuoka, Japan). Because crowding or isolation may be stressful for experimental animals, rats were housed in cages, containing five animals each, throughout the experiment. Rats were fed a standardized diet of hard briquettes and water, and were maintained under a 12-h light/dark cycle (light on 08:00–20:00 h) at a temperature of 22°C and relative humidity of 50%. The body weight of the rats was recorded every 2 d, in the morning after being taken out of restraining cages, from the beginning to the end of the experimental period.

Rats were given sulfamethoxazole (1 mg/ml) and trimethoprim (200 μg/ml) in drinking water, *ad libitum*, for 4 d to reduce the original oral flora, followed by a 4-d antibiotic-free period before being challenged with *P. gingivalis*. Rats were orally challenged with *P. gingivalis* ATCC 33277, which were suspended in 5% carboxymethylcellulose, and each rat received 0.5 ml (1.5×10^{10} cells/ml) by oral gavage (four times) at 48-h intervals. To study the influence of restraint stress, rats were randomly divided into four groups of five animals each. Restraint stress was induced by enclosing each animal in flexible wire mesh (5×5 mm) shaped to fit its body. Animals were unable to move and had no access to food or water. Because rats are nocturnal animals, they were restrained from 20:00 h to 08:00 h (12 h) for the entire experimental period. In the nonrestrained groups (groups A and C), rats were denied food and water for the same time period, but were not restrained. After release from the restrainer, the rats were returned to their home cages each morning. The five rats in each cage belonged to the same group. Group A served as the control group, group B was exposed to restraint stress, group C was orally challenged with *P. gingivalis* and group D was exposed to restraint stress and was orally challenged with *P. gingivalis* (Fig. 1). The experimental procedures of this study were reviewed and approved by the committee of ethics on animal experiments of Kanagawa Dental College and were carried out under the guidelines for animal experimentation of Kanagawa Dental College.

Recovery of *P. gingivalis*

To confirm *P. gingivalis* colonization, plaque samples were collected with a sterile cotton swab from the rat oral cavities. Plaque samples in 100 μl of distilled water were boiled for 10 min, cooled on ice, and then examined by polymerase chain reaction (PCR), as described by Ashimoto *et al*. (19). For identification of *P. gingivalis*, the following primers were used to amplify a 404-bp fragment of the 16S rRNA gene: primer 1 (5′-AGG CAG CTT GCC ATA CTG CG-3′) and primer 2 (5′-ACT GTT AGC AAC TAC CGA TGT-3′). Briefly, 5 μl of sample was added to 45 μl of reaction mixture containing 5 μl of 10 × PCR buffer,

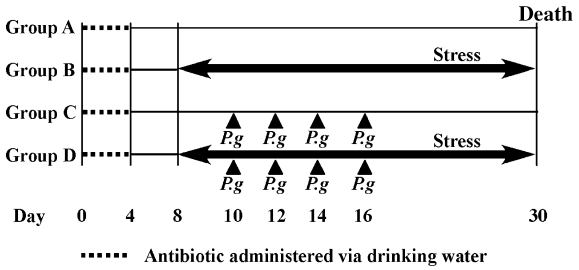

Fig. 1. Experimental procedure. Rats were divided into four experimental groups. Each group included five rats ($n = 5$). Group A, control (nonrestrained and nonchallenged with *Porphyromonas gingivalis*); group B, restrained and nonchallenged with *P. gingivalis*; group C, orally challenged with *P. gingivalis and* nonrestrained; group D, restrained and orally challenged with *P. gingivalis*. *P.g*, *Porphyromonas gingivalis*.

Platinum® *Taq* DNA polymerase and 0.2 mM of each deoxyribonucleotide (Invitrogen Corporation, San Diego, CA, USA). PCR amplification was perfomed in an iCycler™ (Bio-Rad, Laboratories, Hercules, CA, USA) and comprised an initial denaturation step at 95°C for 2 min, followed by 36 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 1 min and an extension at 72°C for 1 min, and then a final elongation step at 72°C for 2 min. Amplicons were detected by electrophoresis of 10 μl of PCR product on a 1.5% agarose gel. Electrophoresis was conducted at 10 V/cm in Tris-Boric acid-EDTA (TBE) buffer. The gel was stained with 0.5 μg/ml ethidium bromide and photographed under 302-nm ultraviolet light. Band sizes were confirmed with reference to molecular size markers (One STEP Ladder 50®; Nippon Gene, Tokyo, Japan).

Laboratory assays

Blood was taken by cardiac puncture under anesthesia (veterinary ketalar® 50, intramuscular) in order to measure corticosterone and cortisol when the rats were killed. Blood samples were centrifuged, separated into serum and kept frozen at −20°C. In the case of corticosterone and cortisol, serum was assayed using a commercial radioimmunoassay kit (Immunotech, Beckman Coulter Co., Fullerton, CA, USA).

Tissue preparation

At the end of the experimental period, all animals were killed by decapitation under anesthesia (veterinary ketalar® 50, intramuscular). The rats were killed in the morning between 08:00 h and 11:00 h. The animals of the four experimental groups were killed immediately after the last restraint session. Tissue blocks containing all three maxillary molars, alveolar bone and surrounding soft tissues were removed from the right side of the maxilla (split-mouth experimental design). The left side of the maxilla was used as a dry specimen for measurement of horizontal alveolar bone loss. The thymus and spleen were also removed, washed in saline and weighed. All tissue samples were fixed in 10% buffered formaldehyde (pH 7.4).

Histological findings

Specimens were decalcified in 10% EDTA for 2 wks and were then dehydrated in graded alcohol, carefully oriented and embedded in paraffin with the axis of the teeth parallel to the cutting direction. Blocks were serially cut in 5-μm sections in the mesial-distal direction. The most central section from each tooth (i.e. the section comprising the center of the dental pulp), was selected for analysis, stained with hematoxylin and eosin (H&E) and mounted.

Alveolar bone loss in rats by *P. gingivalis* infection

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 (p.s.i.), and were then immersed in 3% hydrogen peroxide, rinsed, air-dried and stained with 1% methylene blue. Horizontal bone loss around the maxillary molars was assessed morphometrically. The distance from the cemento–enamel junction (CEJ) to the alveolar bone crest (ABC) was measured at seven palatal sites per rat. Measurements were made under a dissecting microscope (×40) fitted with a digital high-definition system (digital HD microscope, VH-7000; Keyence, Osaka, Japan) that was standardized to give measurements in millimeters.

Reverse transcription–polymerase chain reaction analysis of cytokines

Gingival tissues were dissected from the upper jaws when the rats were killed. Tissues were minced and extracted, and the extracts were purified and concentrated. Total RNA was isolated from the gingival tissue using an RNeasy® Mini Kit, according to the manufacturer's instructions (QIAGEN GmbH, Hilden, Germany). cDNA was reverse transcribed and amplified using the SuperScript™ III One-Step reverse transcription–polymerase chain reaction (RT–PCR) System with Platinum® *Taq* DNA Polymerase (Invitrogen). Based on previous reports (20,21), the following primer pairs (including PCR product size in parentheses) were synthesized by Invitrogen: interleukin-1β, 5′-AAG CTC TCC ACC TCA ATG GAC AG-3′ and 5′-CTC AAA CTC CAC TTT GCT CTT GA-3′(260 bp); tumor necrosis factor-α (TNF-α), 5′-CAC GCT CTT CTG TCT ACT GA-3′ and 5′-GGA CTC CGT GAT GTC TAA GT-3′ (616 bp); IFN-γ, 5′-ATC TGG AGG AAC TGG CAA AAG GAC G-3′ and 5′-CCT TAG GCT AGA TTC TGG TGA CAG C-3′ (288 bp); and β-actin, 5′-TTG TAA CCA ACT GGG ACG ATA TGG-3′ and 5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′ (759 bp). Cycling conditions for denaturation, annealing and extension were as follows: IL-1β, 35 cycles of 94°C for 60 s, 58°C for 45 s and 72°C for 45 s; TNF-α, 35 cycles of 94°C for 60 s, 58°C for 45 s and 72°C for 45 s; IFN-γ, 40 cycles of 94°C for 60 s, 58°C for 45 s and 72°C for 45 s; and β-actin, 35 cycles

of 94°C for 60 s, 58°C for 45 s and 72°C for 45 s. PCR was performed with an iCycler™ (Bio-Rad). PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. An image analyzer (AE-6905 H Image Saver HR; Atto, Tokyo, Japan) was used to detect signal intensity. Band sizes were confirmed with reference to molecular size markers (One STEP Ladder 50®; Nippon Gene). Densitometric analysis was performed on cytokine gene expression by NIH image (NIH, Bethesda, MD, USA). Values for each cytokine mRNA were normalized against the amount of β-actin mRNA, which was utilized as a housekeeping gene for each experimental condition.

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).

Results

Body weight

At the beginning of the experimental period, all rats had a similar body weight. Because the rats were in their growth period, their body weight increased during the experiment. This gain was 35% in the nonrestrained groups (groups A and C) and 25% in the restrained groups (groups B and D). Mean body weight in the restrained groups (groups B and D) was less than that in the nonrestrained groups (groups A and C), and this trend continued until the end of the experimental period ($p < 0.01$) (Fig. 2).

Thymus and spleen

Thymus and spleen weights were significantly lower in the restrained groups (groups B and D) than in the nonrestrained groups (groups A and C) ($p < 0.05$) (Figs 3 and 4).

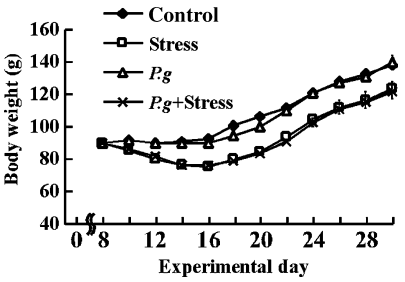

Fig. 2. Change of body weight. The body weights (mean ± standard error) of rats in the restrained groups (groups B and D) were increased significantly less than the body weights of rats in the nonrestrained groups (groups A and C) ($p < 0.01$). *P.g*, *Porphyromonas gingivalis*.

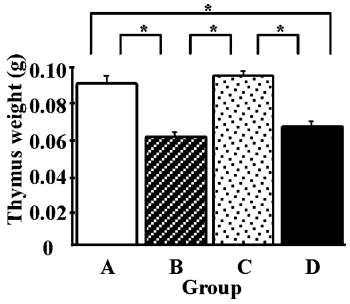

Fig. 3. Effects of stress conditions on the weight of lymphatic organs. Twenty rats were divided into four groups: group A, control (nonrestrained and nonchallenged with *Porphyromonas gingivalis*); group B, restrained and nonchallenged with *P. gingivalis*; group C, orally challenged with *P. gingivalis and* nonrestrained; group D, restrained and orally challenged with *P. gingivalis*. The results are expressed as means ± standard error ($p < 0.05$).

Assays for stress markers

In the restrained groups (groups B and D), corticosterone and cortisol values were significantly higher than those in the nonrestrained groups (groups A and C) ($p < 0.05$) (Table 1).

Alveolar bone loss

In order to determine the role of restraint stress in alveolar bone loss, bone level differences of the rats were determined between the restrained groups and the nonrestrained groups. Figure 5 shows the mean [± standard error of the mean (SEM)] CEJ:ABC value at each of the seven measurement sites. As alveolar bone decreased, the CEJ:ABC increased. In the four experimental groups, CEJ:ABC was greater in the *P. gingivalis*-challenged groups (groups C and D) than in the *P. gingivalis*-nonchallenged groups (groups A and B) at almost every site, indicating bone loss. The distance between the CEJ and ABC increased in the *P. gingivalis*-challenged groups (groups C and D). This distance did not increase in the *P. gingivalis*-nonchallenged groups (groups A and B). In addition, the distance between the CEJ:ABC was greater in the combined restraint stress and *P. gingivalis*-challenged group (group D) than in the *P. gingivalis*-challenged group (group C), and overall alveolar bone loss was greatest in the combined restraint stress and *P. gingivalis*-challenged group (group D) ($p < 0.05$) (Figs 5 and 6D). The recovery of orally infected *P. gingivalis* was checked by the PCR method. *P. gingivalis* was recovered from all *P. gingivalis*-challenged rats at the initiation and the termination of the experiments. No *P. gingivalis* were detected in the noninfected control. The PCR-amplified band did not differ significantly between the restrained groups and the nonrestrained groups (data not shown).

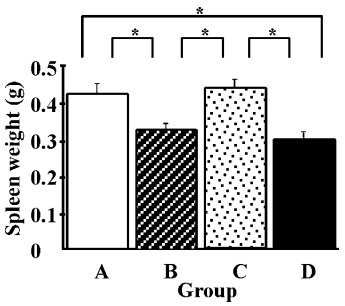

Fig. 4. Effects of stress conditions on weight of lymphatic organs. Twenty rats were divided into four groups: group A, control (nonrestrained and nonchallenged with *Porphyromonas gingivalis*); group B, restrained and nonchallenged with *P. gingivalis*; group C, orally challenged with *P. gingivalis and* nonrestrained; group D, restrained and orally challenged with *P. gingivalis*. The results are expressed as means ± standard error ($p < 0.05$).

Table 1 . Blood corticosterone and cortisol levels

Group	Corticosterone (ng/ml)	Cortisol (mg/dl)
A	380.60 ± 40.38	2.70 ± 0.23
B	526.00 ± 39.41[a]	3.64 ± 0.21[a]
C	356.60 ± 41.10	2.14 ± 0.15
D	584.40 ± 35.10[b]	3.52 ± 0.29[b]

[a]Significantly different ($p < 0.05$) from Groups A and C.
[b]Significantly different ($p < 0.05$) from Groups A and C.
Group A, control (nonrestrained and nonchallenged with *Porphyromonas gingivalis*); group B, restrained and nonchallenged with *P. gingivalis*; group C, orally challenged with *P. gingivalis and* nonrestrained; group D, restrained and orally challenged with *P. gingivalis*.

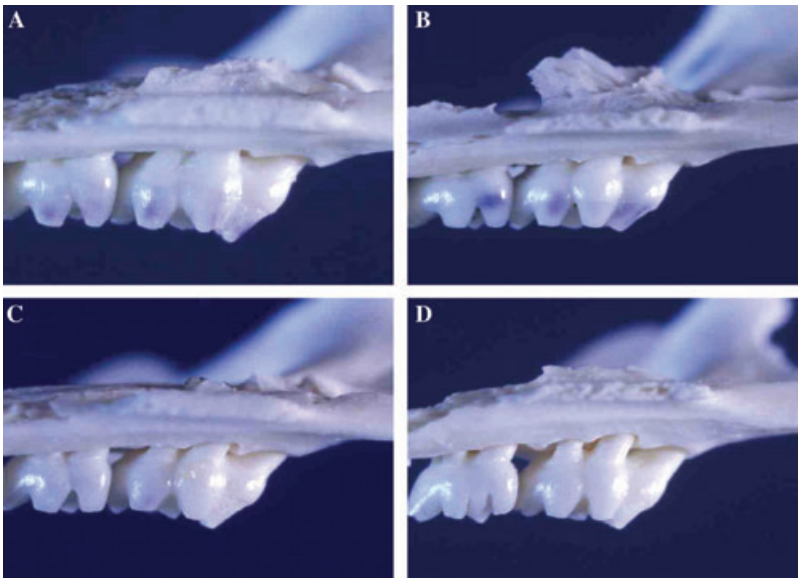

Fig. 5. Morphometric bone levels. Bone loss occurred in rats orally challenged with *Porphyromonas gingivalis* ATCC 33277, but not in the control group (group A) or the restrained group (group B). Columns represent the means of data from five rats (mean ± standard error) ($p < 0.05$). Data from rats exposed to restraint stress and orally challenged with *P. gingivalis* (group D) were different from data from rats orally challenged with *P. gingivalis* (group C).

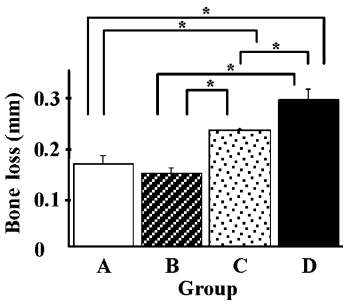

Fig. 6. Alveolar bone loss caused by oral infection with *Porphyromonas gingivalis*. Measurement of bone levels was performed by comparing the distance from the cemento–enamel junction (CEJ) to the alveolar bone crest (ABC) at seven palatal sites on three molars on the left side of the maxilla. (A) Control rat; (B) restraint stress rat; (C) *P. gingivalis*-challenged rat; and (D) restraint stress and *P. gingivalis*-challenged rat. As alveolar bone is resorbed in orally challenged rats, the distance from the CEJ to the ABC increases. Jaws depicted were selected from rats that closely mirror the mean alveolar bone loss values of the respective group.

Thus, restraint stress had no direct effect on bacterial colonization.

Histopathological findings

Sections were placed under a Nikon microscope (Nikon, Tokyo, Japan). Sections from experimental sites were magnified 20 times in order to investigate alveolar bone resorption. The osteoclasts were seen in the *P. gingivalis*-challenged groups (Fig. 7C,D). However, the restraint stress alone produced no notable microscopic changes in periodontal tissue (group B) (Fig. 7B). The osteoclasts were detected significantly on the bone surface near the degenerating tissues in the combined restraint stress and *P. gingivalis*-challenged group (group D) (Fig. 7D).

RT–PCR analysis of cytokine gene expression in gingival tissue

In order to substantiate the functional changes in rat gingival tissue under restraint stress and bacterial infection, the gene expression profiles of interleukin-1β, TNF-α and IFN-γ were compared by RT–PCR. As shown in Fig. 8, cytokine expression was reduced in the restrained groups (groups B and D) when compared with the nonrestrained groups (groups A and C). In the restrained groups (groups B and D), densitometric analysis revealed that the 50% lower in interleukin-1β, TNF-α, and IFN-γ compared with the nonrestrained groups (groups A and C). The relative quantity of the PCR products in the *P. gingivalis*-challenged group (group C) was similar to the levels in the control group (group A).

Discussion

In the present study, we investigated the influence of restraint stress on *P. gingivalis*-challenged periodontitis in an experimental rat model. We found that restraint stress applied over a period of 22 d accelerates the degradation of periodontal tissues and alveolar bone loss. Adult periodontitis is widespread in humans and is associated with the presence of the gram-negative, anaerobic, black-pigmented bacterium represented by *P. gingivalis* (1,22). *P. gingivalis* has been shown to represent a significant proportion of

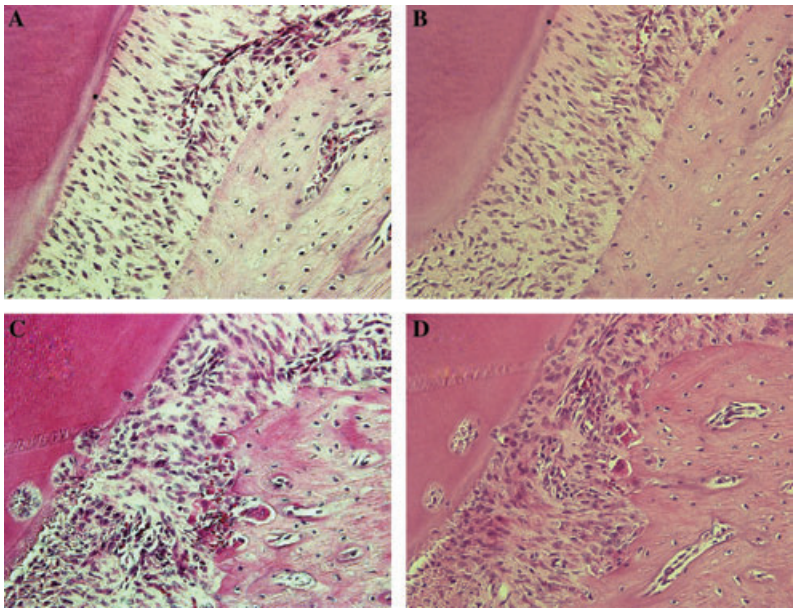

Fig. 7. The periodontal tissues. The osteoclasts were not seen in the rats nonchallenged with *Porphyromonas gingivalis* (A,B). In restraint stress rats (B), stress alone produced no notable microscopic changes in periodontal tissue, However, *P. gingivalis*-challenged rats (C,D), osteoclasts were observed around alveolar bone (hematoxylin & eosin; original magnification ×20) (C,D).

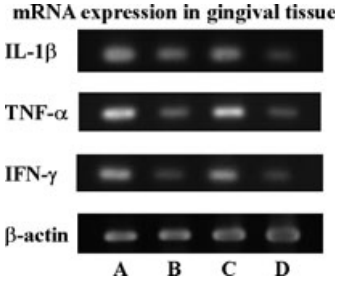

Fig. 8. Comparison of cytokines [(interleukin-1β, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)] expression in gingival tissue by reverse transcription–polymerase chain reaction (RT–PCR). mRNA was extracted from the gingival tissue and analyzed by RT–PCR. PCR products were separated on 2% agarose gels and stained with ethidium bromide. There was a difference between the restrained groups (groups B and D) and the nonrestrained groups (groups A and C); expression was lower in the restrained groups (groups B and D). Representative results of four independent experiments are shown, and the experiment was repeated twice with similar results. β-actin, β-actin was used as a loading control.

the culturable flora in established periodontal lesions and has been implicated in the pathogenesis of periodontal disease. The organism expresses a number of potential virulence factors that have been implicated in the pathogenesis of periodontitis. *P. gingivalis* lipopolysaccharide can activate osteoclasts directly and causes the release of prostaglandin E_2 and the cytokines interleukin-1β and TNF-α from macrophages, monocytes and fibroblasts (23–27). These compounds are potent local mediators of bone resorption and, moreover, can inhibit collagen synthesis by osteoblasts and induce the production of host metalloproteases that destroy connective tissue and bone.

The rat experimental periodontitis model used in this study was a relatively simple *in vivo* model in which a single periodontal organism infects the animal, leading to destructive periodontitis. Therefore, we used a *P. gingivalis*-challenged animal model to examine the influence of restraint stress. Stress has been linked to periodontal disease since the middle of the last century. In the past decade, more evidence has emerged from epidemiological studies relating periodontitis to stress, depression and negative life events (16). Animal studies conducted in the 1960s demonstrated the possible detrimental role of stress in periodontal tissues (17). In a series of studies on rats, Breivik *et al.* (18) demonstrated that periodontal disease susceptibility and progression could be explained, at least in part, by brain-neuroendocrine-immune regulatory mechanisms. Genetically determined hypothalamic–pituitary–adrenal (HPA) reactivity also appears to play an important role, and periodontal disease is likely to send feedback signals to the brain (28). Conventional studies on the influence of restraint stress and periodontitis were performed by tying ligatures around the cervical area of the maxillary molars under anesthesia; however, this method might damage the cervical area of the maxillary molars. To overcome this problem, rats were infected with *P. gingivalis* in the oral cavity, four times at 48-h intervals, without anesthesia. Blood glucocorticoid levels were measured as markers of stress adaptation. In the restrained groups, levels of the hormone markers cortisol and corticosterone, were elevated with involution of the thymus and spleen (Table 1). Thymus weight in the restrained groups was 33% lower than in the nonrestrained groups. Similarly, spleen weight in the restrained groups was 24% lower than in the nonrestrained groups (Figs 3 and 4). These results indicate the physiological burden caused by restraint stress in rats.

The gains of body weight between the restrained groups and the nonrestrained groups were observed at the end of the experiment. In the restrained groups, an overall lower increase in weight was observed (Fig. 2). In comparison with the nonrestrained groups, the restrained groups clearly exhibited inferior development. The combination of restraint stress and *P. gingivalis* challenge (Figs 5 and 6D) resulted in significantly higher attachment and alveolar bone loss than *P. gingivalis* challenge alone (Figs 5 and 6C). However, restraint stress alone did not induce alveolar bone loss (Figs 5 and 6B). Histopathologically, osteoclasts were seen at the interdental septum of alveolar bone (Fig. 7). Shklar &

Glickman (29) observed that stress, without local irritants for periodontal inflammation, produced no notable microscopic changes in the gingiva, periodontal membrane, alveolar bone, or cementum in rats. Gaspersic *et al*. (30) also reported that restraint stress alone had no significant effects on the structure of periodontal tissues. Light microscopy examination of the maxilla in the restrained group, periodontal tissues in the root furcation area of the right first maxillary molar, did not show inflammatory cell infiltration, dilatation of capillaries or disorganization of the gingival fibers in the connective tissue (Fig. 7B). After 22 d, the periodontal tissues revealed that considerable changes in the combined restraint stress and *P. gingivalis*-challenged group had occurred, and osteoclasts were seen on the bone surface near the degenerating tissues after hematoxylin and eosin (H&E) staining (Fig. 7D). In the *P. gingivalis*-challenged periodontitis experimental rat model, some other pathway may activate osteoclasts operative in stimulating bone loss directly, because the PCR products in the group challenged with *P. gingivalis* alone were similar to those in the control group. Moreover, interleukin-1β, TNF-α and IFN-γ were prominent cytokines in infection-stimulated bone resorption, but were not up-regulated under stress conditions. The combined restraint stress and *P. gingivalis*-challenged group also showed a decrease in cytokine expression (Fig. 8). The RT–PCR data suggest that the restraint stress inhibits cell activation and local production of chemoattractant factors. This suggests 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 release of arachidonic acid metabolites and granule-containing enzymes, such as elastase and collagenase, in addition to cytokines such as interleukin-1 and TNF-α is markedly inhibited by glucocorticoid (31,32). Glucocorticoids also inhibit the ability of macrophages to kill various phagocytized microorganisms (33). Moreover, glucocorticoids also reduce the number of tissue macrophages. Functionally, monocytes and macrophages are among the most sensitive cells to the anti-inflammatory effects of glucocorticoids. The results of the present study showed that the restraint stress-induced high values of glucocorticoids, such as cortisol-inhibited production of cytokines, thereby promoting the degradation of periodontal tissues and alveolar bone loss.

Genco *et al*. (32) reported that analysis of the role of stress in infectious processes, such as periodontal disease, should consider the overall effects of stressors appraised by the brain. Stress-induced responses either result in a change in behavior, or are transmitted to the HPA axis to promote the release of corticotropic-releasing hormone (CRH) from the hypothalamus and glucocorticoid from the adrenal cortex. Stress perceived by the brain stimulates the hypothalamus to produce CRH, which is released into the hypophyseal portal system, thus activating the pituitary gland to release adrenocorticotropic hormone (ACTH), which in turn induces release of corticosteroids from the adrenal cortex. Corticosteroids also inhibit the production of cytokines, including interleukin-1, -2, -3, -6, TNF, IFN-γ, and granulocyte–monocyte colony-stimulating factors. Hence, stress-related stimulation of the HPA axis, together with the production of glucocorticoid, such as cortisol, has major suppressive actions on the immune and inflammatory responses.

Our results demonstrated obvious differences in periodontal breakdown in the four experimental groups, particularly with the combination of restraint stress and oral challenged with *P. gingivalis*. We confirmed that restraint stress with periodontal inflammation already present modulates the progression of periodontal disease and alveolar bone loss. Most of the attachment loss in periodontitis is thought to be the result of an inappropriate immune response to dental plaque microorganisms. Furthermore, polymorphonuclear leukocyte degranulation is considered to be an important tissue-destructive mechanism, occurring by the release of proteolytic enzymes, such as the matrix metalloproteinases (MMPs), and by reactive oxygen metabolites during phagocytosis. The local tissue destruction in periodontitis may thus in part be the result of a compensatory high influx of phagocytic polymorphonuclear leukocytes during a glucocorticoid-induced weak T helper 1 immune response to combat and control pathogens in the gingival sulcus (18). Periodontal disease susceptibility may depend on both genetic and environmental factors modulating the HPA responsively. Thus, dental plaque microorganisms may result in destructive disease in individuals with an ability to mount a high glucocorticoid response to gingival inflammation or in subjects under restraint stress resulting in constantly elevated glucocorticoid levels. This notion is supported by a recent epidemiological study showing an association between salivary cortisol levels, stress, distress and coping behaviour, and human periodontal disease (32). Baker *et al*. (34) demonstrated that oral challenge of mice with *P. gingivalis* stimulated oral bone loss and that the observed bone loss occurred in a site-specific manner. Furthermore, it appears that oral bone loss is linked to T-cell activation (35).

A positive correlation between restraint stress and periodontitis was demonstrated in this study. It appears that the dramatic changes occurred in periodontal tissue as a result of restraint stress. However, further study of these observations is needed. We conclude that restraint stress affects and modulates the periodontitis progression. In addition, these findings support the fact that restraint stress may play an important role in periodontal breakdown. To evaluate the relationship between stress and periodontitis, knockout animal studies are needed to examine the effect of cytokine ablation on the course of oral challenge with *P. gingivalis*.

References

1. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol Mol Biol Rev* 1998;**62:**1244–1263.

2. Holt SC, Bramanti TE. Factors in virulence expression and their role in periodontal disease pathogenesis. *Crit Rev Oral Biol Med* 1991;**2:**177–281.
3. Genco RJ. Current view of risk factors for periodontal disease. *J Periodontol* 1996;**67:**1041–1049.
4. Monteiro DA, Silvia AM, Newman NH, Oakley DA, O'Leary R. Psychosocial factors in inflammatory periodontal diseases. *J Clin Periodontol* 1995;**22:**516–526.
5. Breivik T, Thrane PS, Murison R, Gjermo P. Emotional stress effects on immunity, gingivitis and periodontitis. *Eur J Oral Sci* 1996;**104:**327–334.
6. Roberts A, Matthews JB, Socransky SS, Freestone PPE, Williams PH, Chapple ILC. Stress and periodontal diseases: effects of catecholamines on the growth of periodontal bacteria *in vivo*. *Oral Microbiol Immunol* 2002;**17:**296–303.
7. Roberts A, Matthews JB, Socransky SS, Freestone PPE, Williams PH, Chapple ILC. Stress and the periodontal diseases: growth responses of periodontal bacteria to *Escherichia coli* stress-associated autoinducer and exogenous Fe. *Oral Microbiol Immunol* 2005;**20:**147–153.
8. Cohen S. Stress and infections disease in human. *Psychol Bull* 1991;**109:**5–24.
9. Sheridan JF, Dobbs C, Brown D, Zwilling B. Psychoneuro-immunology: stress effect on the pathogenesis and immunity during infection. *Clin Microbiol Rev* 1994;**7:**202–212.
10. Blalock JE. The syntax of immune-endocrine communication. *Immunol Today* 1994;**15:**504–511.
11. Hall NR, Goldstein AL. Endocrine regulation of host immunity. In: Fenichel RL, Chirogos MA, eds. *Immune Modulation Agents and Their Mechanisms*. New York: Marcel Dekker, 1984: 533–563.
12. Singh VK, Warren RP, White RD, Leu SJC. Corticotropin-releasing factor induced stimulation of immune functions. *Ann N Y Acad Sci* 1990;**594:**416–419.
13. Mantyh PW. Substance P and the inflammatory and the immune response. *Ann N Y Acad Sci* 1991;**632:**263–271.
14. Miller SC, Thaller JL, Soberman A. The use of Minnesota multiphasic personality inventory as a diagnostic aid in periodontal disease – a preliminary report. *J Periodontol* 1956;**27:**44–46.
15. Minnerman MA, Cobb C, Soriano F, Burns S, Schuchman L. Relationships of personality traits and stress to gingival status or soft-tissue oral pathology: an exploratory study. *J Public Health Dent* 1995;**55:**22–27.
16. Genco RJ, Ho AW, Grossi SG, Dunford RG, Tedesco LA. Relationship of stress, distress, and inadequate coping behaviors to periodontal disease. *J Periodontol* 1999;**70:**711–723.
17. Gupta OP, Blechman H, Stahl SS. Effect of stress on the periodontal tissues of young adult male rats and hamsters. *J Periodontol* 1960;**31:**413–417.
18. Breivik T, Opstad PK, Gjermo P, Thrane PS. Effects of hypothalamic–pituitary–adrenal axis reactivity on periodontal tissue destruction in rats. *Eur J Oral Sci* 2000;**108:**115–122.
19. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 1996;**11:**266–273.
20. Fukushima A, Ozaki A, Fukata K, Ueno H. Differential expression and signaling of IFN-γ in the conjunctiva between Lewis and brown Norway rats. *Microbiol Immunol* 2003;**47:**785–796.
21. Liu XY, Zhou HF, Pan YL *et al*. Electroacupuncture stimulation protects dopaminergic neurons from inflammation-mediated damage in medial forebrain bundle-transected rats. *Exp Neurol* 2004;**189:**189–196.
22. Slots J, Listgarten MA. *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases. *J Clin Periodontol* 1988;**15:**85–93.
23. Lindemann RA, Economou JS, Rothermel H. Production of interleukin-1 and tumor necrosis factor by human peripheral monocytes activated by periodontal bacteria and extracted lipopolysaccharides. *J Dent Res* 1988;**67:**1131–1135.
24. Roberts FA, Richardson GJ, Michalek SM. Effects of *Porphyromonas gingivalis and Escherichia coli* lipopolysaccharide on mononuclear phagocytes. *Infect Immun* 1997;**65:**3248–3254.
25. Sismey-Durrant HJ, Hopps RM. Effect of lipopolysaccharide from *Porphyromonas gingivalis* on prostaglandin E_2 and interleukin-1β release from rat periosteal and human gingival fibroblasts *in vitro*. *Oral Microbiol Immunol* 1991;**6:**378–380.
26. Wilson MK, Reddi Henderson B. Cytokine-inducing components of periodontopathogenic bacteria. *J Periodont Res* 1996;**31:**393–407.
27. Yamaji Y, Kubota T, Sasaguri K *et al*. Inflammatory cytokine gene expression in human periodontal ligament fibroblasts stimulated with bacterial lipopolysaccharides. *Infect Immun* 1995;**63:**3576–3581.
28. Breivik T, Sluyter F, Hof M, Cools A. Differential susceptibility to periodontitis in genetically selected Wister rat lines that differ in their behavioral and endocrinological response to stressors. *Behav Genet* 2000;**30:**123–130.
29. Shklar G, Glickman I. The periodontium and salivary glands in the alarm reaction. *J Dent Res* 1953;**32:**773–778.
30. Gaspersic R, Stiblar-Martincic D, Skaleric U. Influence of restraint stress on ligature-induced periodontitis in rats. *Eur J Oral Sci* 2002;**110:**125–129.
31. Genco RJ, Ho AW, Kopman J, Grossi SG, Dunford RG, Tedesco LA. Models to evaluate the role of stress in periodontal disease. *Ann Periodontol* 1998;**1:**288–302.
32. Cao L, Martin A, Polakos N, Moynihan JA. Stress causes a further decrease in immunity to herpes simplex virus-1 in immunocompromised hosts. *J Neuroimmunol* 2004;**156:**21–30.
33. Schaffner A. Therapeutic concentrations of glucocorticoids suppress the antimicrobial activity of human macrophages without impairing their responsiveness to gamma interferon. *J Clin Invest* 1985;**76:**1755–1764.
34. Baker PJ, Evans RT, Roopenian DC. Oral infection with *Porphyromonas gingivalis and* induced alveolar bone loss in immunocompetent and severe combined immunodeficient mice. *Arch Oral Biol* 1994;**39:**1035–1040.
35. Baker PJ, Dixon M, Evans RT, Dufour L, Johnson E, Roopenian DC. CD_4 T cells and the proinflammatory cytokines gamma interferon and interleukin-6 contribute to alveolar bone loss in mice. *Infect Immun* 1999;**67:**2804–2809.