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Hydrogen-rich water attenuates experimental periodontitis in a rat model

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Abstract

Aim: Reactive oxygen species (ROS) contribute to the development of periodontitis. As molecular hydrogen can act as a scavenger of ROS, we examined the effects of treatment with hydrogen-rich water on a rat model of periodontitis.

Material & Methods: A ligature was placed around the maxillary molars for 4 weeks to induce periodontitis, and the animals were given drinking water with or without hydrogen-rich water.

Results: The rats with periodontitis which were treated with pure water showed a time-dependent increase in serum ROS level. Compared with the rats without periodontitis, the periodontitis-induced rats which were given pure water also showed polymorphonuclear leucocyte infiltration and alveolar bone loss at 4 weeks. Hydrogen-rich water intake inhibited an increase in serum ROS level and lowered expression of 8-hydroxydeoxyguanosine and nitrotyrosine in the periodontal tissue at 4 weeks. Such conditions prevented polymorphonuclear leucocyte infiltration and osteoclast differentiation following periodontitis progression. Furthermore, inflammatory signalling pathways, such as mitogen-activated protein kinases, were less activated in periodontal lesions from hydrogen-rich water-treated rats as compared with pure water-treated rats.

Conclusion: Consuming hydrogen-rich water might be beneficial in suppressing periodontitis progression by decreasing gingival oxidative stress.

Key words: animal studies; antioxidants; hydrogen-rich water; oxidative stress; periodontitis

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Periodontitis is a chronic inflammatory disease of the supporting tissues of teeth, which is initiated by dental plaque biofilm and its products (Madianos et al. 2005). In the periodontal tissue, host cells such as polymorphonuclear leucocytes produce reactive oxygen species (ROS) as part of the host defence against bacterial pathogens (Chapple & Matthews 2007). However, when ROS overwhelm the cellular antioxidant defence, damage to DNA, proteins and lipids in host tissue also occurs (oxidative stress) (Circu & Aw 2010). Clinical studies have shown that periodontitis is correlated with decreased total antioxidant status and/or increased lipid peroxidation in gingival crevicular fluid, saliva or blood (Akalın et al. 2007, Khalili & Biloklytska 2008, Wei et al. 2010). Animal studies also demonstrated that experimental periodontitis-induced expression of 8-hydroxydeoxyguanosine (8-OHdG) level (an indicator of oxidative DNA damage) and nitrotyrosine (an indicator of oxidative protein damage) in fibroblasts or polymorphonuclear leucocytes (Tomofuji et al. 2006, Ekuni et al. 2008, Maruyama et al. 2011). These observations indicate that oxidative stress is involved in progression of periodontitis.

Molecular hydrogen, which selectively reduces cytotoxic ROS, is considered to be a novel antioxidant

Conflict of interest and source of funding statement

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(Ohsawa et al. 2007). Drinking water containing a therapeutic dose of hydrogen (hydrogen-rich water, HW) represents an alternative mode of delivery of molecular hydrogen. Previous animal studies have demonstrated that the antioxidative effects of HW reduced atherosclerosis in apolipoprotein E knockout mice (Ohsawa et al. 2008), prevented chronic allograft nephropathy after renal transplantation in rats (Cardinal et al. 2010), and improved vitamin C deficiency-induced brain injury in SMP30/GNL knockout mice (Sato et al. 2008). Therefore, it is possible that HW is of potential therapeutic value in the prevention of oxidative stress-related diseases, including periodontitis. However, it is still unclear whether consuming HW offers clinical benefits on periodontal health.

In the present study, we hypothesized that consuming HW may suppress periodontitis progression by decreasing oxidative stress. Thus, the purpose of this study was to investigate the preventive effects of HW on ligature-induced periodontitis in rats. To gain better insight of the mechanism of action, we analysed the histological changes, oxidative tissue damage and inflammatory intracellular signalling pathway activation (most notably activation of mitogen-activated protein kinases, MAPK). Furthermore, the serum level for reactive oxygen metabolites (ROM) (whole oxidant capacity of serum against *N*,*N*-diethylparaphenylendiamine in acidic buffer) was also determined to evaluate the production of ROS in the periodontal lesions (Tamaki et al. 2009, D'Aiuto et al. 2010).

Material and Methods

Animals

Twenty-eight male Wistar rats (8-weeks-old) were housed in an air-conditioned room (23–25°C) with a 12-h light–dark cycle. They had free access to powdered food (MF; Oriental Yeast Co. Ltd., Osaka, Japan) and drinking water. All experimental procedures were performed in accordance with the regulations of the Animal Research Control Committee of Okayama University Dental School.

Experimental design

The rats were randomly divided into four groups of seven rats each: (i) Control group: animals were given pure water and received no treatment; (ii) HW group: animals were given HW and received no treatment; (iii) Periodontitis group: animals were given pure water and experimental periodontitis was induced; or (iv) Periodontitis + HW group: animals were given HW and experimental periodontitis was induced. A 3/0 cotton ligature (Alfresa Pharma Co., Osaka, Japan) was placed in a submarginal position of the maxilla second molars to induce periodontitis (Irie et al. 2008). HW was produced by Blue Mercury Inc. (Tokyo, Japan) using a HW-producing apparatus, by which molecular hydrogen was dissolved in pure water under a pressure of 0.4 MPa, as previously described (Cardinal et al. 2010). The HW (hydrogen concentration; 800–1000 μg/l) was stored in an aluminium bag and placed in a glass vessel twice a day.

After the experimental period, the animals were sacrificed under general anaesthesia. For histological analysis, the left maxillary molar regions were resected en bloc from each rat and were fixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 1 day. Gingival biopsy samples of the right maxillary molar regions were homogenized by the frozen cell crusher (Microtec Co., Chiba, Japan). The homogenized samples from four rats per group were used for measurement of the glutathione level, whereas those from three rats per group were used for the western blot analysis.

Histopathological analysis

After fixation with paraformaldehyde, the maxillary samples were decalcified with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 weeks at 4°C. Formalin-fixed tissue samples were embedded in paraffin following dehydration with ethanol (70%, 80%, 90% and 100%) and immersion in xylene. The paraffin-embedded bucco-lingual 4-μm sections were then stained with haematoxylin and eosin or other stains, as described below.

Immunohistochemical staining for 8-OHdG and nitrotyrosine was performed using commercial kits (Nichirei Co., Tokyo, Japan). The polyclonal antibody against 8-OHdG (Chemicon International, Temecula, CA, USA) (Tomofuji et al. 2006) and nitrotyrosine (Upstate Biotech, DBA, Milan, Italy) (Maruyama et al. 2011) was diluted at 1/200 and 1/50, respectively, in phosphate buffered saline. The colour was developed with 3-3′-diamino bentizine tetrahydrochloride, and sections were counterstained with Mayer's haematoxylin. To identify osteoclasts, tartrate-resistant acid phosphatase (TRAP) activity was also detected using the azo dye method (Sanbe et al. 2009).

A blinded single examiner (T. T.) performed the following histometrical analyses using a light microscope. The distance between the cemento-enamel junction and the alveolar bone crest (a marker of alveolar bone loss) was measured with a microgrid at a magnification of ×200 (Irie et al. 2008). The polymorphonuclear leucocytes in the connective tissue subjacent to the junctional epithelium were counted in two standard areas [0.05 mm (depth) × 0.1 mm each] under a magnification of ×400 (Irie et al. 2008). The numbers of 8-OHdG-positive fibroblasts, nitrotyrosine-positive fibroblasts and total fibroblasts in standard areas (0.1 mm × 0.1 mm each) adjacent to the alveolar bone surface within the periodontal ligament (three serial areas from the top of the alveolar bone crest) were determined (Tomofuji et al. 2006). TRAP-positive osteoclasts occurring along the whole edge of the bone surface were counted and reported as number/millimetres (Sanbe et al. 2009). We evaluated intra-examiner reproducibility by double-scoring 10 randomly selected sections at 2-week intervals. Agreement with one polymorphonuclear leucocyte was more than 90%.

Measurement of gingival glutathione level

The levels of total glutathione (GSH + GSSG level) and GSSG were determined with colorimetric microplate assay kits (Dojindo

Laboratories, Tokyo, Japan). GSSG was determined after blocking GSH with 2-vinylpyridine and the GSH/GSSG ratio (a marker of cellular antioxidant level) in each sample was calculated (Irie et al. 2008).

Western blot analysis

Total proteins were extracted from gingival tissue samples using a detergent-based extraction buffer (Invitrogen, Carlsbad, CA, USA), and sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed using standard protocols. Total and phosphoprotein levels were determined by western blot using primary rabbit polyclonal antibodies and secondary goat anti-rabbit antibodies (1 : 10,000; Pierce Chemical, Rockford, IL, USA). The following primary antibodies were used: anti-phosphorylated-extracellular signal-regulated protein kinase1/2, anti-total-extracellular signal-regulated protein kinase1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phosphorylated-p38 MAP kinases, anti-phosphorylated c-Jun N-terminal kinase, anti-total-p38 and anti-total-c-Jun N-terminal kinase (all from Cell Signaling Technology, Beverly, MA, USA). α-Tubulin was used as internal control. The images of bands were quantified by densitometry using an Epson perfection scanner (Epson Europe, Amsterdam, the Netherlands). Each data point was then normalized relative to the α-tubulin present in that sample (Ekuni et al. 2011).

Measurements of serum ROM

Blood samples were collected from the tail vein at baseline and 2 weeks and from the heart at 4 weeks. Serum was separated by centrifugation at 1500 *g* for 15 min. The level of ROM was determined using the free radical electric evaluator (Diacron International, Grosseto, Italy) (Tamaki et al. 2009, D'Aiuto et al. 2010). The measurement unit was Carratelli Unit (CARR U). One unit of CARR corresponded to 0.08 mg/dl hydrogen peroxide.

Statistical analysis

The differences in histological data and serum ROM level among the four groups were analysed by one-way ANOVA followed by Tukey's method. *T*-test was used for statistical comparison of gingival oxidative stress between the Periodontitis and Periodontitis + HW groups. *p*-value <0.05 was considered statistically significant.

Results

There were no significant differences among the Control, HW, Periodontitis and Periodontitis + HW groups with regard to food consumption, body weight or growth pattern during the experimental period. Addition of hydrogen to drinking water did not change the water intake.

The density of polymorphonuclear leucocytes, the distance between the cemento-enamel junction and alveolar bone crest and the number of TRAP-positive osteoclasts were greater in the Periodontitis group than in the Control and HW groups at 4 weeks ($p < 0.05$) (Table 1, Fig. 1). The densities of polymorphonuclear leucocytes and TRAP-positive osteoclasts in the Periodontitis + HW group showed a 60% and 61% decrease compared with that in the Periodontitis group respectively ($p < 0.05$). The distance between the cemento-enamel junction and alveolar bone crest seemed to be lower in the Periodontitis + HW group than in the Periodontitis group, but this difference was not significant. In addition, the densities of polymorphonuclear leucocytes and TRAP-positive osteoclasts were also greater in the Periodontitis + HW group than in the Control and HW group at 4 weeks ($p < 0.05$).

The ratio of 8-OHdG-positive fibroblasts to total cells in the Periodontitis + HW group demonstrated a decrease of 69% compared with that of the Periodontitis group at 4 weeks ($p < 0.05$) (Fig. 2). The ratio of nitrotyrosine-positive fibroblasts to total cells in the Periodontitis + HW group also demonstrated a decrease of 47% compared with that of the Periodontitis group at 4 weeks ($p < 0.05$). On the other hand, gingival GSH/GSSG ratio of the Periodontitis + HW group exhibited 38% increase compared with that of the Periodontitis group at 4 weeks ($p < 0.05$).

The results of western blot analysis are shown in Fig. 3. MAP kinases, including c-Jun N-terminal kinase, p-38 and extracellular signal-regulated protein kinase, were less activated in periodontal tissues obtained from the Periodontitis + HW group than in those obtained from the Periodontitis group.

Serum levels of ROM in the Periodontitis group increased in a time-dependent manner, and these values were significantly higher than in the Control and HW groups at 2 and 4 weeks ($p < 0.05$) (Fig. 4). Serum levels of ROM in the Periodontitis + HW group were significantly lower than those in the Periodontitis group at 2 and 4 weeks ($p < 0.05$). However, the serum level of ROM in the Periodontitis + HW group was also

Table 1. Histopathological evaluation in periodontal tissues at 4 weeks (mean ± SD)

	Control ($N = 7$)	HW ($N = 7$)	Periodontitis ($N = 7$)	Periodontitis + HW ($N = 7$)
Polymorphonuclear leucocytes (numbers/0.05 mm × 0.1 mm)	0.8 ± 0.3	0.9 ± 0.4	4.0 ± 0.6*†	1.6 ± 0.5*†‡
Distance between the cemento-enamel junction and the alveolar bone crest (μm)	386 ± 57	415 ± 63	605 ± 114*†	498 ± 65
TRAP-positive osteoclasts (numbers/mm)	0.8 ± 0.5	0.4 ± 0.3	5.6 ± 1.3*†	2.2 ± 0.5*†‡

TRAP, tartrate-resistant acid phosphatase.
*Significantly different from the Control group, $p < 0.05$ (Tukey's method).
†Significantly different from the hydrogen-rich water (HW) group, $p < 0.05$ (Tukey's method).
‡Significantly different from the Periodontitis group, $p < 0.05$ (Tukey's method).

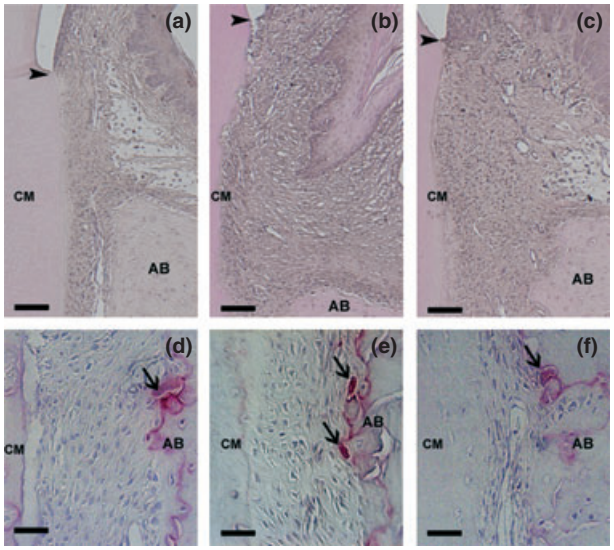

Fig. 1. Haematoxylin and eosin (a–c) or tartrate-resistant acid phosphatase stains (d–f) of rat periodontal tissue at 4 weeks. No pathological changes (a) and little positive tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts (arrows, red-stained cytoplasm) (d) were observed in the periodontium in any of the samples in the Control group. The Periodontitis group showed the distance between the cemento-enamel junction (arrow) and alveolar bone crest (b) and the number of TRAP-positive osteoclasts (e) more than the Control (a and d) and the Periodontitis + HW groups (c and f) respectively. AB, alveolar bone; CM, cementum. Scale bar = 100 μm (a–c) and 30 μm (d–f) respectively.

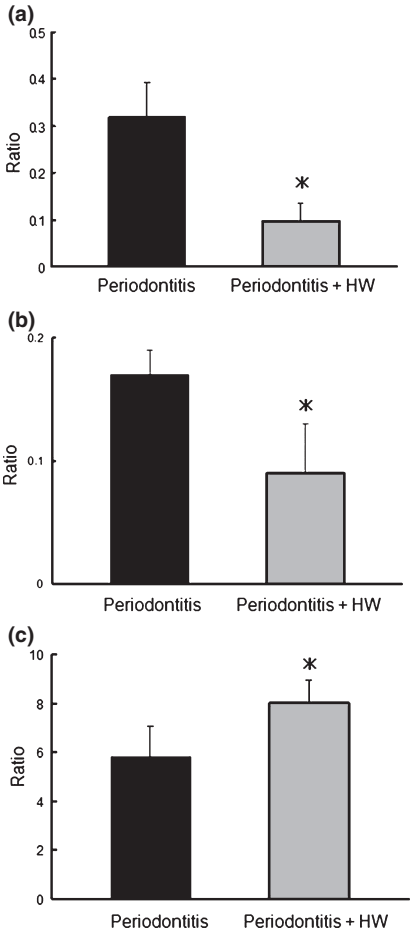

Fig. 2. The ratios of 8-hydroxydeoxyguanosine (8-OHdG) positive fibroblasts ($n = 7$) (a), nitrotyrosine-positive fibroblasts ($n = 7$) (b) and GSH/GSSG ($n = 4$) (c) in the periodontal tissue at 4 weeks (mean ± SD). *$p < 0.05$, compared with the Periodontitis group (*t*-test).

significantly higher than the serum level in the Control and HW groups at 4 weeks ($p < 0.05$).

Discussion

To our knowledge, this is the first study demonstrating the preventive effects of HW on experimental periodontitis in rats. In this study, ligature placement resulted in alveolar bone loss and polymorphonuclear leucocyte infiltration at 4 weeks. This observation indicates that the ligature placement induced experimental periodontitis. In addition, the consumption of HW decreased polymorphonuclear leucocyte infiltration in the periodontal lesions at 4 weeks. Furthermore, lower expressions of 8-OHdG and nitrotyrosine and higher GSH/GSSG ratio were found in the rats with periodontitis and HW consumption than in the rats with periodontitis at 4 weeks. 8-OHdG, nitrotyrosine and GSH/GSSG ratio are accepted as parameters of oxidative DNA damage (Tomofuji et al. 2006), oxidative protein damage (Maruyama et al. 2011) and cellular antioxidant status (Irie et al. 2008) respectively. It is conceivable that HW could reduce periodontitis-induced oxidative stress, and this effect might suppress periodontal inflammation.

In the rats with periodontitis, serum level of ROM increased in a time-dependent manner. As ROM is considered to be a reliable indicator of circulating ROS (Tamaki et al. 2009, D'Aiuto et al. 2010), this suggests that excessive production of ROS in the periodontal lesions occurred following periodontitis progression. On the other hand, HW suppressed the increase in serum ROM level following periodontitis at 2 and 4 weeks. Consuming HW appears to reduce production of ROS in the periodontal lesion. Cytotoxic ROS is involved in progression of periodontitis (Chapple & Matthews 2007). Diminished cytotoxic ROS by HW consumption would contribute to the reduction of gingival oxidative stress.

Studies have shown the antioxidative effects of HW on various organs. For instance, significantly less superoxide formation in the brain was observed in the HW consumption group than in the pure water consumption group in vitamin C-depleted SMP30/GNL knockout mice (Sato et al. 2008). Moreover, myocardial 8-OHdG concentration in irradiated mice was significantly lower in the HW-treated groups than in the controls (Qian et al. 2010). These findings are consistent with the present results showing that HW decreased gingival 8-OHdG and nitrotyrosine expressions and serum ROM level in rats with periodontitis.

Reactive oxygen species can activate MAPK pathways (Torres & Forman 2003), which play an essential role in inflammatory osteolysis (Rogers et al. 2007, Wei & Siegal 2007). In this study, HW reduced the number of TRAP-positive osteoclasts with a decreased protein expression of MAPK. It is possible

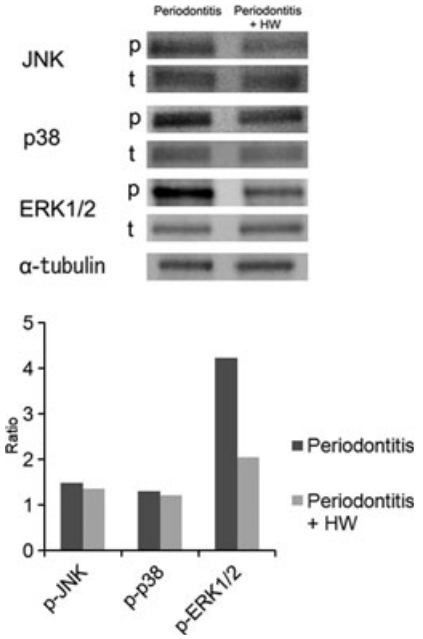

Fig. 3. Gingival protein expression of mitogen-activated protein kinases (MAPK) (JNK, p38 and ERK1/2) in the Periodontitis and Periodontitis + HW groups. p, phosphorylated; t, total. The α-tubulin was used as internal control. Bars represent the ratio of each protein expression to the α-tubulin.

that HW decreased osteoclast differentiation by suppressing the MAPK pathways.

However, we found that the rats with periodontitis and HW consumption, exhibited more TRAP-positive osteoclasts and polymorphonuclear leucocytes than the control rats. These findings show that suppression of periodontitis by HW was incomplete. In this study, we applied HW without removing the ligature, suggesting that the suppression of periodontitis by HW occurred under the condition in which dental plaque formation was not removed.

A clinical study demonstrated that the intake of grapefruit led to an increase in plasma vitamin C concentration and improved gingival bleeding in periodontitis patients (Staudte et al. 2005). Animal studies also have demonstrated that supplementation of cocoa polyphenols (Tomofuji et al. 2009), *N*-acethylcysteine (a thiol antioxidant) (Toker et al. 2009), proanthocyanidins (a flavanoid extracted from grape seeds) (Govindaraj et al. 2010) and green tea catechins (Maruyama et al. 2011) induced a significant reduction in periodontal inflammation. Furthermore, a review suggests that adequate daily intake of natural antioxidants is recommended for the prevention and treatment of periodontitis (Van der Velden et al. 2011). These evidences support the concept that antioxidant therapy would offer clinical benefits in improvement and prevention of periodontitis. In this study, we found that the antioxidative effects of HW exerted beneficial effects in suppression of periodontitis. Molecular hydrogen is continuously produced by colonic bacteria in the body and normally circulates in the blood (Reth 2002), indicating that the side effect of molecular hydrogen might be small and different from the other antioxidants. Therefore, HW may have great potential for clinical use.

When HW was applied in the stomach, hydrogen was detected in the blood (Nagata et al. 2009), suggesting that molecular hydrogen could be incorporated into the body by drinking. Molecular hydrogen diffuses very rapidly into tissues. The concentration of molecular hydrogen increased within 15 min. after application, and then returned to the baseline level (Cardinal et al. 2010). In this study, general anaesthesia was performed to obtain periodontal tissue samples. As periodontal tissue samples were obtained more than 15 min. after HW consumption, we did not determine the concentration of molecular hydrogen in periodontal tissue samples.

Some antioxidants may have the antibacterial effects in the periodontium. For instance, it is known that green tea catechins showed a bactericidal effect against black-pigmented, Gram-negative anaerobic rods in periodontal pockets (Hirasawa et al. 2002). Therefore, molecular hydrogen may also have the beneficial effects on periodontal health by its antibacterial actions. However, further studies are needed to clarify this issue.

We used the ligature-induced periodontitis model in this study. This model induces acute periodontal inflammation that is not directly equivalent to chronic periodontitis in humans. Therefore, further studies are needed to evaluate the effects of HW on chronic periodontitis in humans. This is a limitation of our study.

In conclusion, consuming HW could diminish periodontitis-induced cytotoxic ROS and gingival oxidative stress, which in turn might suppress periodontal inflammation and osteoclast differentiation on alveolar bone.

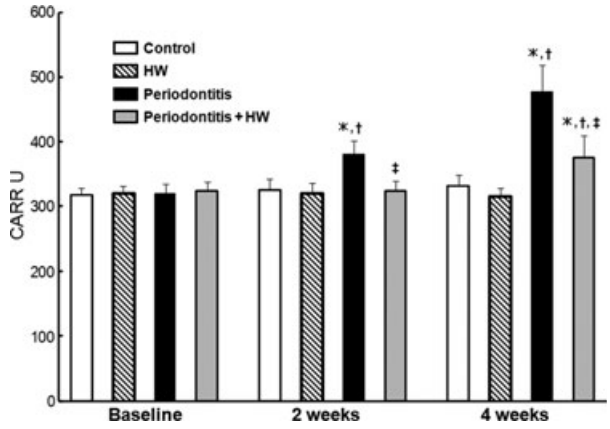

Fig. 4. Serum reactive oxygen metabolites (ROM) level at baseline, 2 or 4 weeks. Values are presented as means of seven rats ± SD. $*p < 0.05$, compared with the Control group (Tukey's method). $^{\dagger}p < 0.05$, compared with the hydrogen-rich water (HW) group (Tukey's method). $^{\ddagger}p < 0.05$, compared with the Periodontitis group (Tukey's method).

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Clinical Relevance

Scientific rationale for the study: Molecular hydrogen is considered to be a novel antioxidant. It is possible that hydrogen-rich water (HW) is of potential therapeutic value in the prevention of oxidative stress-related diseases, including periodontitis. However, it is still unclear whether consuming HW offers clinical benefits on periodontal health.

Principal findings: Hydrogen-rich water intake inhibited an increase in serum reactive oxygen species and lowered expression of 8-OHdG and nitrotyrosine in the periodontal lesion.

Practical implications: Hydrogen-rich water could reduce periodontitis-induced oxidative stress, and this effect might suppress periodontal inflammation.