Hydrogen-rich water attenuates experimental periodontitis in a rat model


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.

Conflict of interest and source of funding statement

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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 (Akalin 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.
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 aluminum 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% tetradsodium-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 benzidine 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 Materials & 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 Committee of Okayama University Dental School.

Material and Methods

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TRAP-positive osteoclasts (numbers/mm) 0.8 ± 0.5

et al. 2011). Tubulin present in that sample (Ekuni was then normalized relative to the
the Netherlands). Each data point
scanner (Epson Europe, Amsterdam,

Western blot analysis
Total proteins were extracted from
gingival tissue samples using a deter-
gent-based extraction buffer (Invitro-
gen, Carlsbad, CA, USA), and
sodium dodecyl sulphate polyacryl-
amide gel electrophoresis was per-
formed using standard protocols.
Total and phosphoprotein levels
were determined by western blot
using primary rabbit polyclonal anti-
body and secondary goat anti-rab-
bit antibodies (1 : 10,000; Pierce
Chemical, Rockford, IL, USA). The
following primary antibodies were
used: anti-phosphorylated-extracellu-
lar signal-regulated protein kinase1/2,
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 densi-
tometry 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 centrifu-
gation at 1500 g for 15 min. The level
of ROM was determined using the
free radical electric evaluator (Dia-
cron 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 statisti-
cal 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, Periodonti-
tis and Periodontitis + HW groups
with regard to food consumption,
body weight or growth pattern dur-
ing the experimental period. Addi-
tion of hydrogen to drinking water
did not change the water intake.

The density of polymorphonu-
clear leucocytes, the distance
between the cemento-enamel junc-
tion and alveolar bone crest and the
number of TRAP-positive osteo-
clasts were greater in the Periodonti-
tis 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 junc-
tion and alveolar bone crest seemed
to be lower in the Periodontitis
+ HW group than in the Periodonti-
tis 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 Peri-
donitis + 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, gingi-
vial GSH/GSSG ratio of the Peri-
donitis + HW group exhibited
38% increase compared with that
of the Periodontitis group at 4 weeks
(p < 0.05).

The results of western blot analy-
sis are shown in Fig. 3. MAP kinases,
including c-Jun N-terminal kinase,
p-38 and extracellular signal-regu-
lated protein kinase, were less activ-
ated in periodontal tissues obtained
from the Periodontitis + HW group
than in those obtained from the Peri-
donitis group.

Serum levels of ROM in the Peri-
donitis 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). How-
ever, the serum level of ROM in the
Periodontitis + HW group was also

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

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

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).

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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, ligation placement resulted in alveolar bone loss and polymorphonuclear leucocyte infiltration at 4 weeks. This observation indicates that the ligation 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
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-acetylcysteine (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.

References

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.