Hydrogen-rich water prevents lipid deposition in the descending aorta in a rat periodontitis model

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ABSTRACT

Objective: Periodontitis has been causally linked to atherosclerosis, which is mediated by the oxidative stress. As hydrogen-rich water (HW) scavenges reactive oxygen species (ROS), we hypothesized that HW could prevent lipid deposition induced by periodontitis in the aorta. The aim of this study was to investigate the effects of HW on the initiation of atherosclerosis in a rat periodontitis model.

Design: Eighteen 8-wk-old male Wistar rats were divided into three groups of six rats; the periodontitis group, periodontitis + HW group and the no treatment (control) group. In the periodontitis and periodontitis + HW groups, periodontitis was induced using a ligature for 4 wk, while the periodontitis + HW group was given water containing 800–1000 μg/L hydrogen during the 4-wk experimental period.

Results: In the periodontitis group, lipid deposition in the descending aorta was observed. The periodontitis group also showed significantly higher serum levels for ROS and oxidised low-density lipoprotein-cholesterol (ox-LDL) (1.7 and 1.4 times, respectively), and higher aortic expression levels of nitrotyrosine and hexanoyl-lysine (HEL) (7.9 and 16.0 times, respectively), as compared to the control group (p < 0.05). In the periodontitis + HW group, lipid deposition was lower. Lower serum levels of ROS and ox-LDL (0.46 and 0.82 times, respectively) and lower aortic levels of nitrotyrosine and HEL (0.27 and 0.19 times, respectively) were observed in the periodontitis + HW group than in the periodontitis group (p < 0.05).

Conclusions: HW intake may prevent lipid deposition in the rat aorta induced by periodontitis by decreasing serum ox-LDL levels and aortic oxidative stress.

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1. Introduction

Atherosclerosis is a progressive disease characterised by the accumulation of lipid deposits in macrophages (foam cells) in large and medium arteries. Oxidised low-density lipoprotein-cholesterol (ox-LDL) plays a major role in the development and progression of atherosclerosis and its complications. The ox-LDL is formed by oxidative stress and leads to endothelial activation and injury resulting in an inflammatory response that leads to recruitment, activation and migration of monocytes through inter-endothelial gaps to the sub-endothelial region. Studies have demonstrated many atherosclerotic risk factors that induce oxidative stress in the vessel wall,
including smoking, diabetes mellitus, dyslipidemia, hypertension, and periodontitis, although the mechanisms leading to atherosclerosis initiation are not fully understood.

Periodontitis is one of the most widespread inflammatory diseases, and it induces excessive production of reactive oxygen species (ROS) in the periodontal lesion. A number of studies have suggested that there is an association between cardiovascular disease and periodontitis, and that periodontitis plays an etiological role in cardiovascular diseases, including atherosclerosis. A consensus between the American Journal of Cardiology and the Journal of Periodontology was also published in 2009. Furthermore, a clinical study demonstrated that periodontitis causes a systemic increase in ROS and ox-LDL. Based on this evidence, it is feasible that intervention trials are needed in order to identify how antioxidant therapy may have an impact on the atherosclerosis induced by periodontitis.

Molecular hydrogen, which selectively reduces cytotoxic ROS and oxidative stress, is considered to be a novel antioxidant. Drinking water containing a therapeutic dose of hydrogen (hydrogen-rich water; HW) represents an alternative mode of delivery for molecular hydrogen. A previous animal study demonstrated that HW reduces atherosclerosis in apolipoprotein E knockout mice. Therefore, it is possible that HW is of potential therapeutic value in the prevention of atherosclerosis induced by periodontitis.

In the present study, we hypothesized that HW intake suppresses periodontitis-induced aortic oxidative stress by decreasing circulating ox-LDL and preventing the initiation of atherosclerosis. The purpose of this study was to investigate the effects of HW intake on serum ox-LDL levels and lipid deposition in the descending aorta in a rat periodontitis model. In this study, hexanoyl-lysine (HEL) (marker of early stages of lipid peroxidation), nitrotyrosine (marker of protein nitration), and 8-hydroxydeoxyguanosine (8-OHdG) (marker of oxidative DNA damage) were used to evaluate aortic oxidative stress. In addition, the level of reactive oxygen metabolites (ROM) (whole oxidant capacity of serum against N, N-diethylparaphenyldiamine in acidic buffer) was determined as a marker of circulating ROS levels.

2. Materials and methods

2.1. Animals

Eighteen male Wistar rats (age, 8 wk) 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 fresh drinking water. All experimental procedures were performed in accordance with the Animal Research Control Committee of Okayama University.

2.2. Experimental design

Rats were allocated randomly using a random number table to one of three groups (one control and two experimental groups). The control group received distilled water instead of active treatment for 4 wk. In the periodontitis and periodontitis + HW groups, a 3/0 cotton ligature (Alfresa Pharma Co., Osaka, Japan) was placed in a sub-marginal position around the mandibular first molars for 4 wk to induce periodontitis.

The rats in the periodontitis group received distilled water for the 4-wk study period, while the periodontitis + HW group received HW for 4 wk. HW was produced by Blue Mercury Inc. (Tokyo, Japan) using an HW-producing apparatus, by which molecular hydrogen was dissolved in pure water under a pressure of 0.4 MPa, as described previously. 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 4-wk experimental period, animals were sacrificed under general anaesthesia and blood samples were collected from the heart to measure serum levels of HEL, ROM, and ox-LDL. For histological analysis, the mandibular first molar regions were resected en bloc from each rat and fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 1 d. The descending aorta was harvested, immediately frozen, and kept at −80 °C until processing for immunohistochemical analysis or enzyme-linked immunosorbent assay (ELISA).

2.3. Analysis of periodontal tissues

After fixing with paraformaldehyde, mandibular first molar samples were decalcified with 10% tetrasodium–EDTA aqueous solution (pH 7.4) for 2 wk at 4 °C. Formalin-fixed tissue samples were embedded in paraffin following dehydration with ethanol (70%, 80%, 90%, and 100%) and immersion in xylene. Bucco-lingual 4-µm sections embedded in paraffin were stained with haematoxylin and eosin. Immunohistochemical staining for nitrotyrosine was performed using Histofine Simple Stain MAX PO kit (Nichirei Co., Tokyo, Japan) to assess oxidative damage. The polyclonal antibody against nitrotyrosine (Upstate Biotech, DBA, Milan, Italy) was diluted at 1:50 in phosphate buffered saline, followed by treatment with a secondary antibody (Fab) with peroxidase complex for 30 min. Colour was developed with a solution of 3,3′-diaminobenzidine tetrahydrochloride in 50 mmol/L Tris–HCl buffer (pH 7.5) containing 0.001% hydrogen peroxide and sections were counterstained with Mayer’s haematoxylin.

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×. The number of 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.

2.4. Measurements of serum ROM, ox-LDL and lipopolysaccharide (LPS)

Blood samples were allowed to clot at room temperature, and serum was separated by centrifugation at 1500 × g for 15 min. Levels of ROM were determined using a free radical evaluator (Diacon International, Grosseto, Italy) according to the previously reported analysis procedures. Data are given in terms of Carratelli Unit (CARR U), with 1 CARR U corresponding to 0.08 mg/dL hydrogen peroxide. In addition, serum ox-LDL was measured using a commercial ELISA kit for rats (Cusabio Biotech Co., Ltd., Wuhan, China). The level of serum LPS was
also determined using a kinetic limulus amebocyte lysate test kit (Wako Pure Chemical Industries, Osaka, Japan).

2.5. Histological evaluation of aorta samples

Frozen sections (8 μm) were obtained from the descending aorta, embedded in Optimal Cutting Temperature compound (Tissue Tec; Miles, Naperville, IL) and stained with oil red O to detect lipids. Immunohistochemical staining for nitrotyrosine or HEL was performed using Histofine Simple Stain MAX PO kits (Nichirei Co.). After fixation with acetone, sections were then treated at 4°C with an anti-nitrotyrosine (Upstate Biotech) (diluted 1:1000) or anti-HEL (Japan Institute for the Control of Aging, Shizuoka, Japan) (diluted 1:50) overnight, followed by treatment with a secondary antibody (Fab) with peroxidase complex for 30 min. Colour was developed with a solution of 3,3'-diaminobenzidine tetrahydrochloride in 50 mmol/L Tris–HCl buffer (pH 7.5) containing 0.001% hydrogen peroxide and 0.03% hydrogen peroxide. The images were captured using an Axio Imager M2 microscope (Zeiss, Germany) and analysed using AxioVision (Zeiss, Germany) software. The density of polymorphonuclear leukocytes and the ratio of nitrotyrosine-positive fibroblasts to total cells were greater in the periodontitis group than in the control and periodontitis + HW groups. Scale bar = 50 μm (A–C) or 25 μm (D–F). CT, connective tissue; d, dentine; JE, junctional epithelium. *p < 0.017, compared with control group, according to Kruskal–Wallis test, followed by Bonferroni correction of Mann–Whitney U-test. †p < 0.017, compared with periodontitis group, according to Kruskal–Wallis test, followed by Bonferroni correction of Mann–Whitney U-test.

Fig. 1 – Histological view and nitrotyrosine expression of periodontal tissues. No pathological changes are evident in the control group (A and D). The periodontitis group (B and E) showed infiltration of polymorphonuclear leukocytes (arrows) in the connective tissue adjacent to the junctional epithelium. The density of polymorphonuclear leukocytes and the ratio of nitrotyrosine-positive fibroblasts to total cells were greater in the periodontitis group than in the control and periodontitis + HW groups (G and H). Scale bar = 50 μm (A–C) or 25 μm (D–F). CT, connective tissue; d, dentine; JE, junctional epithelium. *p < 0.017, compared with control group, according to Kruskal–Wallis test, followed by Bonferroni correction of Mann–Whitney U-test. †p < 0.017, compared with periodontitis group, according to Kruskal–Wallis test, followed by Bonferroni correction of Mann–Whitney U-test.
sections were counterstained with Mayer’s haematoxylin. Control sections included buffer alone or nonspecific purified rabbit immunoglobulin G. The percentage of the area of total aortic lumen occupied by lipids per section, nitrotyrosine- or HEL-positive aortic lumen was calculated using computer-assisted image analysis software (WinROOF, Mitani Co., Fukui, Japan).³

### 2.6. Measurement of aortic 8-OHdG levels

Aortic samples were homogenized by the Cryo-Press (Microtec Corporation, Chiba, Japan). Mitochondrial DNA was isolated using a DNA extractor kit (Wako, Osaka, Japan) from the precipitate of the homogenates. Concentrations of 8-OHdG were determined using an ELISA kit (High sensitivity; Japan Institute for the Control of Aging).³ Assays were performed in triplicate.

### 2.7. Statistical analysis

All data analysis was performed using a statistical software package (PASW Statistics 8.0 for Windows; IBM, Tokyo, Japan). The differences among the three groups were analysed using the Kruskal–Wallis test and the Mann–Whitney U-test, using the Bonferroni correction to adjust the probability (p < 0.05/3 = 0.017).³ Sample size was calculated using the nQuery Advisor (Statistical Solutions, Saugus, MA), based on the results of our previous study.³ A sample size of 6 per group was required for detection of a significant difference (80% power, two-sided, 5% significance level).

### 3. Results

No significant differences in weight gain or food consumption were detected among the groups during the experimental period. Addition of hydrogen to drinking water did not affect water intake.

There were no pathological changes in the periodontal tissue in the control group (Fig. 1A and D). All periodontal tissue samples in the periodontitis group exhibited infiltration by inflammatory cells, including polymorphonuclear leukocytes (Fig. 1B and E). In all rats in the periodontitis + HW group, infiltration of inflammatory cells was less evident than in the periodontitis group (Fig. 1C and F). The density of polymorphonuclear leukocytes and the ratio of nitrotyrosine-positive fibroblasts to total cells were greater in the periodontitis group than in the control and periodontitis + HW groups (p < 0.017) (Fig. 1G and H).

The serum levels of ROM and ox-LDL in the periodontitis group were significantly higher than those in the control and periodontitis + HW groups (p < 0.017) (Table 1). There was no significant difference in serum level of LPS between three groups.

Lipid deposition in the descending aorta was observed in all rats in the periodontitis group and in none of the rats in the control or periodontitis + HW groups (Fig. 2). The percentage [mean (standard deviation)] of aortic lumen occupied by the lesion was 2.5 (0.7) in the periodontitis group. In addition, nitrotyrosine and HEL expression of the aortic lumen was significantly higher in the periodontitis group compared to the control and periodontitis + HW groups (p < 0.017) (Figs. 3 and 4). On the other hand, aortic 8-OHdG levels in the periodontitis group were significantly higher than those in the control group.

![Fig. 2 - Descending aorta stained with oil red O to show lipid deposition. Lipid deposition (arrowhead) was observed in the periodontitis group (B) and in none of the control (A) or periodontitis + HW (C) group rats. Scale bar = 50 µm.](image)

Table 1 – Difference in serum markers between control, periodontitis and periodontitis + HW groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Periodontitis</th>
<th>Periodontitis + HW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive oxygen metabolites (CARR U)</td>
<td>329 ± 19ᵃ</td>
<td>474 ± 42ᵇ</td>
<td>376 ± 38ᵇ</td>
</tr>
<tr>
<td>Oxidised low density lipoprotein (ng/ml)</td>
<td>66.0 ± 16.7</td>
<td>90.2 ± 7.9ᶜ</td>
<td>74.4 ± 7.0ᶜ</td>
</tr>
<tr>
<td>Lipopolysaccharide (pg/ml)</td>
<td>52.8 ± 18.9</td>
<td>57.8 ± 19.2</td>
<td>50.2 ± 20.9</td>
</tr>
</tbody>
</table>

HW: hydrogen-rich water.
ᵃ Data are expressed as mean ± SD (n = 6).
ᵇ p < 0.017 (between control group and periodontitis group, according to Kruskal–Wallis test, followed by Bonferroni’s correction of Mann–Whitney U-test).
ᶜ p < 0.017 (between periodontitis group and periodontitis + HW group, according to Kruskal–Wallis test, followed by Bonferroni’s correction of Mann–Whitney U-test).
Fig. 3 – Nitrotyrosine expression in cross sections of the descending aorta. Nitrotyrosine expression in the endothelial tissue (arrowhead) of the periodontitis group (B) was more intense than in the control (A) and periodontitis + HW groups (C). The negative control, stained without primary antibody for nitrotyrosine, did not show any nitrotyrosine-positive areas in the descending aorta (D). The percentage of nitrotyrosine-positive lumen (mean ± SD) in the periodontitis group was significantly higher than that in the control and periodontitis + HW groups (E). Scale bar = 50 μm. a p < 0.017, compared with control group, according to Kruskal–Wallis test, followed by Bonferroni correction of Mann–Whitney U-test. b p < 0.017, compared with periodontitis group, according to Kruskal–Wallis test, followed by Bonferroni correction of Mann–Whitney U-test.

(p < 0.017), but not in the periodontitis + HW group (p = 0.025) (Fig. 5).

4. Discussion

In this study, the periodontitis + HW group showed less gingival polymorphonuclear leucocyte infiltration and aortic lipid deposition than the periodontitis group. Serum levels of ROM and ox-LDL, and the degree of nitrotyrosine and HEL formation in the aorta were also lower in the periodontitis + HW group than in the periodontitis group. These findings support the hypothesis that HW attenuates both gingival inflammation and the degree of aortic lipid deposition in the rat periodontitis model with decreasing serum ROS, serum ox-LDL and aortic oxidative stress.

Ox-LDL plays a major role in the initiation and progression of atherosclerosis, showing numerous effects on vascular smooth muscle cells, changing their migration and proliferation activities, and altering their phenotype to foam cells. ROS enhances oxidative modification of LDL to ox-LDL. Ox-LDL also results in generation of ROS from a variety of cell types and contributes to oxidative stress. HW selectively reduces cytotoxic ROS and oxidative stress. In this study, serum levels of ROM and ox-LDL, and aortic oxidative stress were decreased after HW intake. These results suggest that HW attenuates circulating ROS, which may lead to inhibition of ox-LDL production and decreased aortic oxidative stress.

Aortic nitrotyrosine expression was significantly increased by induction of periodontitis and decreased by HW intake in the present study. In another rat model, ischemia-induced cardio-renal injury induced upregulation of nitrotyrosine...
expression and this was inhibited by HW intake in the heart,\textsuperscript{28} which may support the present effects of HW on nitrotyrosine expression. Numerous studies have demonstrated protein nitrination in human atherosclerotic tissues, and that this is associated with different stages of atherosclerosis and is correlated with plaque instability in humans,\textsuperscript{29–31} as well as in rats.\textsuperscript{8} Nitrotyrosine directly increases aortic smooth muscle cell migration in vitro and may contribute to atherosclerosis.\textsuperscript{32} HW may play an important role in improving atherosclerosis by decreasing nitrotyrosine expression.

Lipid peroxidation is involved in the development of atherosclerosis. There is an increase in the levels of serum malondialdehyde as a marker of lipid peroxidation in patients with atherosclerosis when compared to those of controls.\textsuperscript{33} HW reduces aortic lipid peroxidation in apolipoprotein E knockout mice.\textsuperscript{34} In the present study, increased aortic lipid peroxidation, i.e., HEL levels, in the periodontitis group were reduced by HW. HW may contribute to improving atherosclerosis with decreasing lipid peroxidation.

The levels of oxidative DNA damage, i.e., 8-OHdG, were elevated in human atherosclerotic plaques.\textsuperscript{35} In rats, 8-OHdG levels in gastrocnemius are markedly increased by atrophy; however, significant reductions were not observed after HW intake.\textsuperscript{35} HW protects against myocardial degeneration from radiation-induced injury and decreases 8-OHdG in mice.\textsuperscript{36} In this study, aortic 8-OHdG levels in the periodontitis + HW groups were not significantly lower than those in the periodontitis group, but a trend was observed ($p = 0.025$). Further study is required to investigate whether HW decreases up-regulated 8-OHdG levels in periodontitis, although the discrepancy between our results and previous studies may depend on the species (rats vs. mice).

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Fig. 4 – Hexanoyl-lysine (HEL) expression in cross sections of the descending aorta. Hexanoyl-lysine (HEL) expression in the endothelial tissue (arrowheads) of the periodontitis group (B) was more intense than in the control (A) and periodontitis + HW groups (C). Negative control, stained without primary antibody for HEL, did not show any HEL-positive areas in the descending aorta (D). Scale bar = 50 μm. The percentage of HEL-positive lumen (mean ± SD) in the periodontitis group was significantly higher than that in the control and periodontitis + HW groups (E). \textit{a} $p < 0.017$, compared with control group, according to Kruskal–Wallis test, followed by Bonferroni correction of Mann–Whitney U-test. \textit{b} $p < 0.017$, compared with periodontitis group, according to Kruskal–Wallis test, followed by Bonferroni correction of Mann–Whitney U-test.

Our results suggest that HW contributes to reduced cardiovascular disease risk following periodontitis. According to a consensus between the American Journal of Cardiology and the Journal of Periodontology, patients with periodontitis should be informed that it can increase the risk of atherosclerosis, and patients with periodontitis and a history of cardiovascular disease are recommended to undergo a medical examination. However, intervention trials are still needed to identify how periodontal interventions can impact cardiovascular diseases. Adding HW intake to traditional periodontal therapies may be beneficial for periodontitis patients with cardiovascular disease.

Our study has some limitations. We did not investigate bacterial infection in this study, but there was no difference in serum level of LPS between the control and periodontitis groups. A direct and/or indirect mechanism involving bacteria may play a role in the pathological changes of the descending aorta in the present study because bacterial infection contributes to progression of ligature-induced periodontal inflammation. However, the present and previous results support the hypothesis that local inflammation causes an enhanced inflammatory response at distant sites without the spread of the infectious agent and that oxidative stress by periodontitis is an initiating factor leading to the inflammatory injury in the early stages of atherosclerosis. Second, it is unclear whether HW directly improved ROS formation in endothelial cells and/or indirectly attenuated the level of ROS derived from periodontitis in this study. Further study is therefore required to investigate the detailed action mechanisms.

In conclusion, HW intake suppressed the levels of serum ROS and ox-LDL, the degree of lipid deposition in the aorta, and the degrees of nitrotyrosine and HEL formation in the aorta in rat ligature-induced periodontitis. HW intake may prevent aortic lipid deposition induced by periodontitis by decreasing serum ox-LDL and aortic oxidative stress.

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**Competing interests**

There is no conflict of interest related to this research.

**Ethical approval**

The Animal Research Control Committee of Okayama University approved this study. OKU-2010005.

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