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

Daisuke Ekuni [a], *Takaaki Tomofuji* [a,*], *Yasumasa Endo* [a], *Kenta Kasuyama* [a], *Koichiro Irie* [a], *Tetsuji Azuma* [a], *Naofumi Tamaki* [b], *Shinsuke Mizutani* [a], *Azusa Kojima* [a], *Manabu Morita* [a]

[a] *Department of Preventive Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan*

[b] *Department of Preventive Dentistry, The University of Tokushima Institute of Health Biosciences, Graduate School 3-18-15 Kuramoto-cho, Tokushima 770-8504, Japan*

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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 significant 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.

1. Introduction

Atherosclerosis is a progressive disease characterised by the accumulation of lipid deposits in macrophages (foam cells) in large and medium arteries.[1] Oxidised low-density lipoprotein-cholesterol (ox-LDL) plays a major role in the development and progression of atherosclerosis and its complications.[2] 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.[3] Studies have demonstrated many atherosclerotic risk factors that induce oxidative stress in the vessel wall,

* *Corresponding author*. Tel.: +81 86 235 6712; fax: +81 86 235 6714.
E-mail address: tomofu@md.okayama-u.ac.jp (T. Tomofuji).

Abbreviations: HW, hydrogen-rich water; ROS, reactive oxygen species; ox-LDL, oxidised low-density lipoprotein-cholesterol; HEL, hexanoyl-lysine; 8-OHdG, 8-hydroxydeoxyguanosine; ROM, reactive oxygen metabolites; CARR U, Carratelli Unit.

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including smoking,[4] diabetes mellitus,[5] dyslipidemia,[6] hypertension[7,8] and periodontitis,[9,10] 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.[9–12] 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.[13–17] A consensus between the *American Journal of Cardiology* and the *Journal of Periodontology* was also published in 2009.[18] Furthermore, a clinical study demonstrated that periodontitis causes a systemic increase in ROS and ox-LDL.[19] 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.[20] 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.[21] 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),[22] nitrotyrosine (marker of protein nitration),[23] and 8-hydroxydeoxyguanosine (8-OHdG) (marker of oxidative DNA damage)[24] were used to evaluate aortic oxidative stress. In addition, the level of reactive oxygen metabolites (ROM) (whole oxidant capacity of serum against N, N-diethylparaphenylendiamine in acidic buffer) was determined as a marker of circulating ROS levels.[25]

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.[10] 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.[26] 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 leukocytes 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×.[26] 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.[26]

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 (Diacron International, Grosseto, Italy) according to the previously reported analysis procedures.[25] 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