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Therapeutic efficacy of infused molecular hydrogen in saline on rheumatoid arthritis: A randomized, double-blind, placebo-controlled pilot study ☆

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ABSTRACT

The aim of this study was to demonstrate the safety and efficacy of H_2-saline infusion for treatment of rheumatoid arthritis (RA). We conducted a randomized, double-blind, placebo-controlled investigation of the infusion of 1 ppm H_2-dissolved saline (H_2-saline) in 24 RA patients. Patients were randomized 1:1 to receive 500 ml of either H_2-saline or placebo-saline, which was drop infused intravenously (DIV) daily for 5 days. The disease activity score in 28 joints (DAS28) was measured at baseline, immediately post infusion, and after 4 weeks. Therapeutic effects of H_2-saline on joint inflammation were estimated by measuring serum biomarkers for RA, tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), matrix metalloproteinase-3 (MMP-3), and urinary 8-hydroxydeoxyguanosine (8-OHdG). In the H_2-infused group, average DAS28 decreased from 5.18 ± 1.16 to 4.02 ± 1.25 immediately post infusion and reached 3.74 ± 1.22 after 4 weeks. No significant decrease in DAS28 was observed in the placebo group throughout the study. IL-6 levels in the H_2 group significantly decreased in 4 weeks by 37.3 ± 62.0% compared to baseline, whereas it increased by 33.6 ± 34.4% in the placebo group. TNFα levels did not change remarkably in the H_2 or placebo groups in 4 weeks post-infusion compared to baseline. The relative ratio of 8-OHdG in the H_2 group also significantly decreased by 4.7%. After 4 weeks, MMP3 was significantly reduced by 19.2% ± 24.6% in the H_2 group, and increased by 16.9% ± 50.2% in the placebo group. Drop infusion of H_2 safely and effectively reduced RA disease activity.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects approximately 1% of the population. RA is characterized by irreversible joint damage accompanied by destruction of bone and cartilage. In addition, the chronic inflammation associated with RA often damages the skin, subcutaneous tissue, and lungs. Moreover, inflammatory reactions in the arterial endothelium, which occur independently of atherosclerosis risk, promote endothelial dysfunction and increase the risk of cardiovascular disease, thereby diminishing quality of life and survival time [1,2]. Although the etiology is unknown, RA is certainly associated with autoimmune disorders, and its pathogenesis has been well investigated. Auto-reactive T cells that infiltrate the synovial tissue promote the immune response and lead to overproduction of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6). Thus, early RA therapy is based on aggressive biologic modification of the disease through controlling synovial T cells and/or suppressing the levels of cytokines implicated in the disease [3,4]. In addition to these immunogenic factors, reactive oxygen species (ROS) are also important therapeutic targets because they are upstream of the cytokine-mediated inflammatory cascades. Activation of nuclear factor (NF)-κB by excess ROS production leads to increased production of pro-inflammatory cytokines, thereby creating a positive feedback loop and promoting sustained RA inflammation [5]. Hydroxyl radicals, a particular ROS, are harmful because of their rapid and indiscriminate reactivity, and they are thought to play a certain role in the pathogenesis of RA [6–8]. Therefore, clinically effective scavengers that can remove ROS, including hydroxyl radicals, which are surplus to physiological or biological requirements, are expected to emerge as therapeutic agents.

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Because of their extremely high reactivity, hydroxyl radicals react with molecular hydrogen (H_2) through transfer of an electron [9,10]. On the other hand, before being recognized as a medical gas, H_2 has long been used to prevent decompression gas emboli caused by the formation of N_2 bubbles during deep sea diving; therefore, the safety of H_2 has been previously established [11–13]. In contrast to general drugs, no harmful effects of H_2 are observed, even at high concentrations. The potential role of H_2 as an ROS scavenger was first demonstrated by Gharib et al. using a mouse model of schistosomiasis infection-induced chronic liver inflammation [9]. Then, the therapeutic potential of H_2 gas and H_2-enriched water was demonstrated in a rat model of reperfusion injury [14]. In this previous study, it was demonstrated that H_2 scavenges peroxynitrite as well as hydroxyl radicals in cultured cells and in cell-free experiments. Since then, numerous studies concerning the therapeutic potential of H_2 against various diseases related to ROS have been published [10,15]. Recently, we reported that H_2 has therapeutic potential for treatment of RA [16]. In the study, RA patients drank 500 ml of water containing approximately 5 ppm H_2 (high H_2 water) daily for 8 weeks. Oxidative stress was effectively reduced, and RA disease activity was significantly improved. The H_2 absorbed from the water was effective to complement conventional RA therapy, especially in patients in early stages of disease progression or among patients negative for antibodies against cyclic citrullinated peptides (ACPA). However, it is not currently clear how to effectively expose the joints with synovitis or the circulating immune-related cells, which are responsible for the chronic inflammation of RA, to H_2. Moreover, it is not known whether intake of high H_2 water, by which H_2 is thought to passively diffuse through the stomach, is the most effective method for administering H_2. Additional methods for H_2 intake should be investigated, especially as it has the potential to be a rapid, powerful, and safe anti-inflammatory therapeutic strategy. One of the methods for H_2 administration is drop infusion of H_2-dissolved saline (H_2-saline). The aim of this study was to demonstrate the therapeutic potential and safety of H_2-saline for treatment of RA.

2. Materials and methods

2.1. Patients and study design

We conducted a randomized, double-blind, placebo-controlled investigation, and 26 patients who fulfilled the 2010 American College of Rheumatology criteria for RA were enrolled. To verify the safety of intravenous H_2-saline infusion and to confirm the absence of acute infusion reactions and allergic reactions, which may be masked in patients with an inflammatory disease, 20 healthy volunteers were also enrolled from June 2011. All patients and volunteers gave their informed consent to enroll in the study, and this study was approved by the Haradoi Hospital Ethics Committee.

To prepare the H_2-saline, 250 ml of saline in a soft bag was placed in a water bath in which water circulates continuously so as to contain 1.6 ppm H_2 generated by an electrolysis instrument (non-destructive hydrogen-adding apparatus, MiZ Company, Fujisawa, Yokohama, Japan); approximately 1 ppm H_2 was dissolved in the saline before the infusion. Details describing the concentration of H_2 in the saline as generated by the instrument are described elsewhere [17,18], and we also confirmed the concentration by using the methylene blue-platinum colloid regent-based titration method [19]. Placebo-saline was prepared in the same water bath without H_2. Patients were randomized 1:1 to receive 500 ml of either H_2-saline (12 patients) or placebo-saline (12 patients) administered by intravenous drop infusion (DIV) before meal in the morning every day for 5 days. Infusion of each 250 ml bag took approximately 20 min, and the total DIV took approximately 40 min.

The disease activity score 28 (DAS28) based on C-reactive protein (CRP) levels is an estimation of clinical response that is measured by changes in the disease activity score in 28 joints, using C-reactive protein levels [20]. DAS28 levels were estimated about 30 min before starting the first DIV (baseline level), immediately post-infusion (on the 6th–8th day after the baseline), and in 4 weeks from baseline. In addition, fasting blood and urine samples were collected at each point. DAS28 results were classified as good (DAS28 > 1.2), moderate ($0.6 <$ DAS28 ≤ 1.2), or no response (DAS28 ≤ 0.6), according to the European League Against Rheumatism response criteria.

With regard to the safety study, 20 healthy volunteers were randomized 1:1 to the H_2 group (mean age: 53.9 years [range, 28–84]) or placebo group (mean age: 51.8 years [range, 25–93]). They were infused with 500 ml of saline with or without H_2 every day for 3 days. All of the conditions for the solution preparations were the same as the RA study. The influences of H_2-saline on the healthy volunteers were determined by general symptoms or complaints, and blood samples were collected at baseline and 1 week following baseline.

2.2. Biochemical analysis

Serum levels of matrix metalloproteinase-3 (MMP3) were assayed by the latex coagulating nephelometry method using anti-human MMP-3 mouse monoclonal antibody at Mitsubishi Chemical Medience Inc. (Tokyo, Japan). ACPA levels in serum and urinary 8-OHdG in RA patients were estimated by ELISA using MESACUP-2 test CCP (cyclic citrullinated peptides) and the New 8-OHdG Check ELISA kit, respectively, at Mitsubishi Chemical Medience Inc. (Tokyo, Japan). Serum cytokines, TNFα and IL-6, were measured using Millipore's MILLIPLEX Human Cytokine kit by Filgen Inc. (Nagoya, Japan).

2.3. Statistical analyses

Data are presented as the mean ± standard deviation. Analysis of variance was used to compare changes in variables between the H_2-saline and placebo-saline groups. The DAS28 scores at baseline, immediately post-infusion, and in 4 weeks post-infusion were analyzed. The significance of differences in variables (IL-6, TNFα, MMP3, and CRP levels) immediately post-infusion or in 4 weeks from baseline was determined as a ratio of the value at each point to the baseline value. Statistical significance was set at $p < 0.05$. All analyses were performed using SAS v9.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Patient characteristics

All of the data from the 20 healthy volunteers were within standard values, and no patients in either the H_2 or placebo group complained of any adverse effects or changes in general conditions, including flushing, nausea, bradycardia, tachycardia, hyper- or hypotension, pale, dizziness, diarrhea, pain, and general discomfort. No acute infusion reactions or allergic reactions, which could be masked in case of inflammatory diseases like RA, were observed. The biochemical and blood cell data are shown in Table 1.

Among the RA patients, 2 patients who were randomized to receive placebo-saline withdrew from the study in the first 5 days (day 3); 1 withdrew for a personal reason, and the other preferred oral administration. The remaining 24 patients (4 males, 20 females; mean age: 65 years [range, 26–85]) completed the study. The demographic data for RA patients at baseline are shown in Table 2.

ACPA, a part of the EULAR classification criteria for RA [21], were found in 6 patients in the H_2 group and 4 patients in the placebo group. The median disease duration was 4 years and 7 months (range, 6 months to 13 years), and 3 patients in each group had early RA (disease duration < 12 months). None of the patients with early RA had received any medication prior to the study. Five patients in the H_2 group and 4 patients in the placebo group with disease duration >12 months also had not received any medication prior to the study. Three patients

Table 1
Biochemical and blood cell data from healthy volunteers.

	Placebo (n = 10)		H_2 (n = 10)	
	Baseline	1 week	Baseline	1 week
TP, g/dl	7.15 ± 0.367	7.68 ± 0.383	7.15 ± 0.577	7.48 ± 0.299
ALB, g/dl	4.29 ± 0.284	7.75 ± 0.150	4.13 ± 0.393	4.50 ± 0.141
LD, IU/l	151 ± 30.8	142 ± 18.1	161 ± 23.89	153 ± 9.93
AST, IU/l	20.0 ± 2.79	22.8 ± 3.70	21.1 ± 5.32	19.2 ± 1.60
ALT, IU/l	16.5 ± 4.65	21.3 ± 2.49	16.0 ± 5.93	14.4 ± 3.38
ALP, IU/l	185 ± 28.9	205 ± 25.1	195 ± 26.3	205 ± 22.8
GGT, IU/l	22.6 ± 7.61	23.0 ± 5.52	22.0 ± 10.4	18.8 ± 8.28
CK, IU/l	106 ± 44.8	115 ± 33.6	116 ± 62.2	117 ± 43.7
BUN, mg/dl	13.1 ± 4.20	14.3 ± 3.37	13.5 ± 2.52	12.6 ± 2.39
Cr, mg/dl	0.79 ± 0.18	0.78 ± 0.11	0.83 ± 0.15	0.88 ± 0.098
UA, mg/dl	5.29 ± 0.986	4.68 ± 0.59	5.47 ± 0.713	5.14 ± 0.136
TBL, mg/dl	0.77 ± 0.14	0.90 ± 0.16	0.76 ± 0.22	0.76 ± 0.14
ALD, IU/l	3.42 ± 0.722	3.86 ± 0.307	2.90 ± 0.671	2.85 ± 0.541
GLU, mg/dl	97.4 ± 10.1	95.0 ± 6.50	92.05 ± 8.11	94.00 ± 7.00
WBC, cells/ml	5640 ± 1360	4420 ± 1460	5270 ± 1030	4730 ± 477
RBC, $\times 10^4$ cells/ml	491 ± 45.8	513 ± 49.0	450 ± 64.2	482 ± 33.4
Hb, g/dl	14.7 ± 1.26	15.1 ± 1.29	14.0 ± 1.91	14.1 ± 2.01
CRP, mg/dl	0.06 ± 0.037	0.08 ± 0.043	0.10 ± 0.095	0.07 ± 0.030

Data are presented as the mean ± standard deviation. No statistically significant changes were observed between baseline and 1 week post-infusion in either placebo or H2 group. TP, total protein; ALB, albumin; LD, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; CK, creatine kinase; BUN, blood urea nitrogen; Cr, creatinine; UA, uric acid; TBL, total bilirubin; ALD, aldolase; GLU, glucose; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; CRP, C-reactive protein.

in each group had been treated with methotrexate (MTX). One patient in the H_2 group and 2 patients in the placebo group had been treated with bucillamine and glucocorticoid, respectively. None of the 24 patients started additional disease-modifying anti-rheumatic drugs (DMARDs) and/or biological drugs during the study.

3.2. Improvement in disease activity

Regarding the patients with RA, the mean improvements in DAS28 in the H_2 and placebo groups are shown in Fig. 1a. In the 12 H_2-infused patients, DAS28 decreased from 5.18 ± 1.16 to 4.02 ± 1.25 immediately post-infusion and to 3.74 ± 1.22 after 4 weeks. A good DAS28 response was observed in 7 patients, and a moderate response was observed in 4 patients. Although 1 patient showed no response, the scores of all 12 patients decreased at each measurement period. However, DAS28 was not reduced in the 12 patients of placebo group. Eleven patients showed no DAS28 response, and 1 patient had a moderate response. In 4 weeks, the H_2 group showed significant improvement in disease activity compared with the placebo group ($p < 0.01$).

A correlation between reduction in DAS28 by H_2 treatment and ACPA was observed. Six patients in the H_2-infused group and 4 patients in the placebo group had increased ACPA levels (189 ± 164 μ/ml and 92.5 ± 106 μ/ml, respectively). However, 6 patients in the H_2 group and 8 patients in the placebo group showed no increase in ACPA levels. To compare the therapeutic effects of H_2 between the ACPA-negative and ACPA-positive patients, improvements in DAS28 were presented as the difference in DAS28 scores from baseline, as shown in Fig. 1b.

Table 2
Demographic data for RA patients at baseline.

	Placebo (n = 10)	H_2 (n = 10)
Age	68.2 ± 12.6	62.4 ± 18.4
Sex	M, 2; F, 10	M, 2; F, 10
Duration of disease	4 years 3 months	4 years 11 months
DAS28	5.18 ± 1.16	5.10 ± 0.96

Data for age and DAS28 are presented as the mean ± standard deviation.

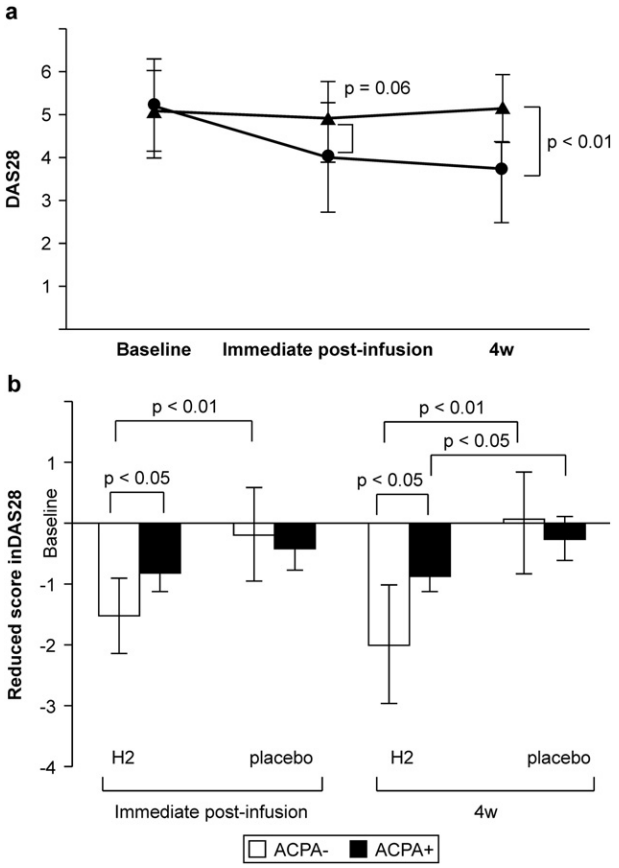

Fig. 1. a. Disease activity scores in 28 joints (DAS28) at baseline, immediately post-infusion, and in 4 weeks (4 w). Error bars represent the mean and standard deviation for measurements in 12 patients in the H_2-infused group (closed circles) or 12 patients in the placebo group (closed triangles). b. The difference in DAS28 from baseline to immediately post-infusion or in 4 weeks. There were 6 patients positive for antibodies against cyclic citrullinated peptides (ACPA) (black columns) in the H_2-infused group and 4 ACPA-positive patients in the placebo group. There were 6 ACPA-negative patients (gray columns) in the H_2-infused group and 8 in the placebo group. Error bars represent the mean ± standard deviation.

Immediate post-infusion, DAS28 reduced significantly in ACPA-negative patients ($p < 0.01$). In 4 weeks after baseline, it was significantly reduced in H_2 group compared to the placebo group, both with ACPA-positive patients ($p < 0.05$) and with ACPA-negative patients ($p < 0.01$). The degree of the decrease in DAS28 in the H_2 group was larger in ACPA-negative patients than in ACPA-positive patients, immediately post-infusion ($p < 0.05$) and in 4 weeks after baseline ($p < 0.05$).

3.3. Effect of H_2-saline infusion on TNFα, IL-6, MMP3, CRP, and urinary 8-OHdG

To investigate whether the antioxidant properties of H_2 affected serum levels of mediators in the ROS-mediated inflammatory chain, which regulates and promotes the production of pro-inflammatory cytokines, TNFα and IL-6 levels were measured. IL-6 was measured in the serum from 9 patients in each group, and TNFα was measured in the serum from 11 patients in the H_2 group and 12 patients in the placebo group. Serum IL-6 at baseline was in the range of 0.24–372 (56.4 ± 120 pg/ml) in the H_2 group and between 6.31 and 456 (90.3 ± 158 pg/ml) in the placebo group. As shown in Fig. 2, in the H_2 group, the relative ratio of IL-6 in 4 weeks to baseline was significantly lower ($p < 0.05$) compared to the ratio in the placebo group. Serum TNFα at baseline was ranged from 8.55 to 366 (55.6 ± 99.9 pg/ml) in the H_2 group and from 7.79 to 2550 (233 ± 730 pg/ml) in the placebo group; however, there was no remarkable change in the relative ratio of TNFα in 4 weeks to baseline between the H_2 group and the placebo

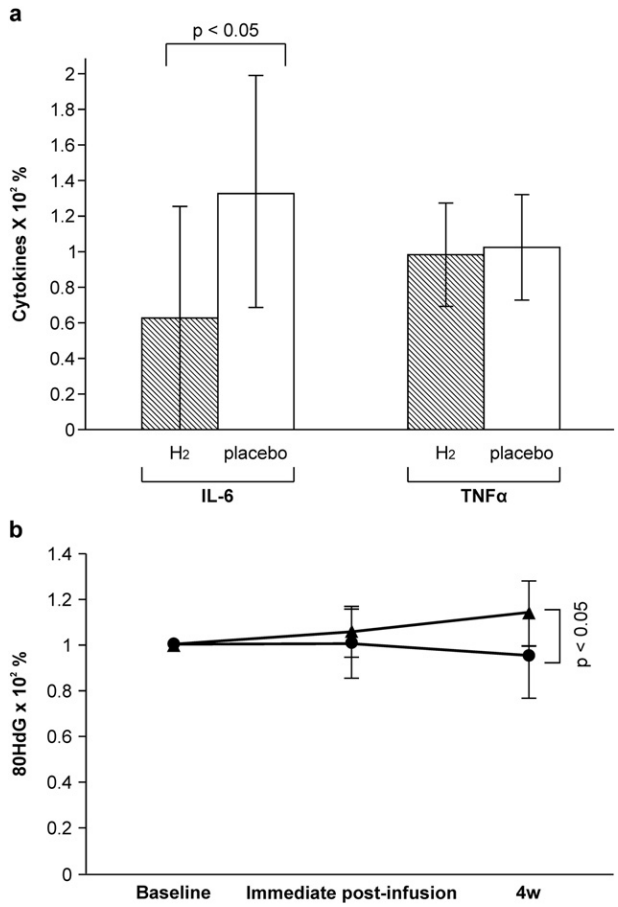

Fig. 2. a. The relative ratios of levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) in 4 weeks to baseline in the H_2 group (gray columns) and placebo group (open columns) are represented. Error bars represent the mean ± standard deviation (closed bars: H_2 group; open bars: placebo group). Two samples in the H_2 group and 3 samples in the placebo group at baseline were below the level of the detection limit for IL-6 (<0.05 pg/ml) and were not added to the data. The serum sample for both cytokines in 4 weeks was not collected from 1 patient in the H_2 group because the patient declined collection of blood for the analysis of cytokine levels due to a fragile vein. b. Urinary 8-hydroxydeoxyguanine (8-OHdG), which ranged from 5.2 to 25.6 ng/mg Cr, is represented as relative ratios ($\times 10^2$%) between immediate post-infusion or in 4 weeks and the concentration at baseline (closed circles: H_2 group; closed triangles: placebo group). Error bars represent the mean and standard deviation (SD).

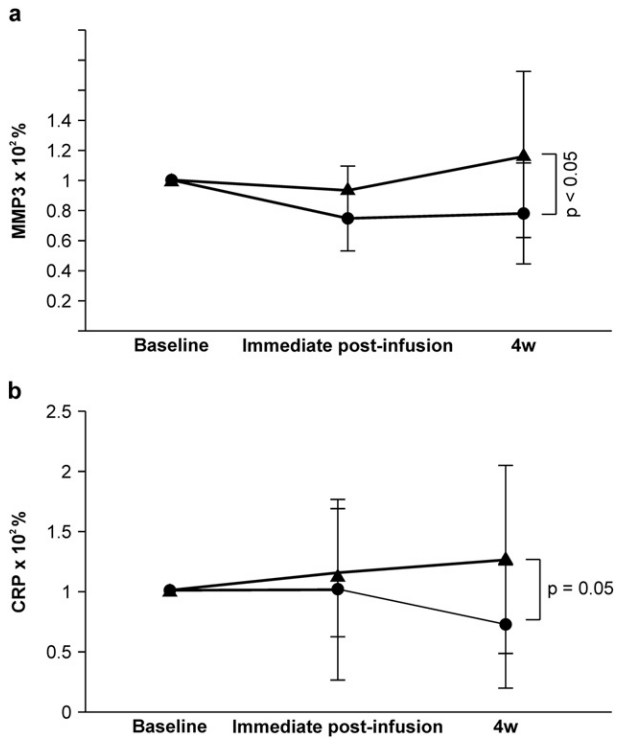

Fig. 3. a. Matrix metalloproteinase-3 (MMP3) levels ranged from 31.3 ng/ml to 2054 ng/ml and are represented as the relative ratios of the concentration in the serum (ng/ml) immediately post-infusion or in 4 weeks to the concentration at baseline (closed circles: H_2 group; closed triangles: placebo group). Error bars represent the mean ± standard deviation. b. The relative ratio ($\times 10^2$%) of the concentration of C-reactive protein (CRP) immediately post-infusion or in 4 weeks to the baseline concentration (closed circles: H_2 group; closed triangles: placebo group). Error bars represent the mean ± standard deviation.

group. The mean relative ratio of urinary 8-OHdG immediately post-infusion and in 4 weeks from baseline are presented in Fig. 2b. Urinary 8-OHdG was significantly decreased in the H_2-infused group compared to placebo group at only 4 weeks post-infusion.

As shown in Fig. 3a, levels of MMP3, which is thought to be an important marker of joint destruction in RA, were measured at baseline (H_2 group: range, 28.4 to 287 ng/ml; mean 106 ± 91.2 ng/ml; placebo group: range, 31.3 to 2,054 ng/ml; mean 280 ± 566 ng/ml), immediately post-infusion and in 4 weeks. The relative ratio between MMP3 levels immediately post-infusion and at baseline decreased by 21.1 ±19.5% in the H_2 group and by 6.3% ± 16.0% in the placebo group ($p = 0.0553$). In 4 weeks after baseline, the relative ratio decreased by 19.2% ± 24.6% in the H_2 group and increased by 16.9% ± 50.2% in the placebo group, thereby demonstrating the significant anti-inflammatory potential of H_2 ($p < 0.05$). In the H_2 group, the serum concentration of CRP (mg/dl) also decreased from 1.47 ± 1.77 at baseline to 1.13 ± 1.39 immediately post-infusion and to 0.83 ± 1.29 in 4 weeks; however, in the placebo group, CRP levels increased from 1.30 ± 2.00 at baseline to 1.41 ± 2.50 immediately post-infusion and to 1.60 ± 2.87 in 4 weeks. As shown in Fig. 3b, the ratio of CRP levels in 4 weeks to baseline decreased in the H_2 group while it increased in the placebo group ($p = 0.05$). The decrease in CRP by the infusion of H_2 saline is consistent with the decline in MMP3 levels observed at the end of the study.

4. Discussion

In the present study, a 5-day infusion of H_2-enriched saline improved disease activity in RA patients more effectively in 4 weeks than immediately post-infusion, as confirmed by the reduction of MMP3 levels. This indicates that the therapeutic potential of H_2 administered intravenously is observed with a delay of 3 weeks. This finding is consistent with our previous study, in which patients drank 500 ml of water containing 4–5 ppm H_2 daily for 4 weeks and RA disease activity was significantly improved. H_2 seems to have a constitutive efficacy, even during the 4-week washout period when the patients did not drink H_2 water. This effect cannot be explained by the continuous elimination of hydroxyl radicals. In the present study, urinary 8-OHdG levels were reduced after the delay of the washout period. Based on these 2 studies, the mechanisms by which H_2 reduces RA-induced inflammation seem to be related not only to direct scavenging of hydroxyl radicals but also to another indirect process related to autoimmune responses.

One such indirect mechanism of action is thought to be through NF-κB modulation. As depicted in Fig. 4, the inflammation-related positive feedback loops are attributed to NF-κB action and are induced by redox imbalance [5]. Therefore, excess ROS and NF-κB, which are located at the center of the feedback loops, up-regulate pro-inflammatory cytokines, including TNFα and IL-6, thereby activating nicotinamide adenine dinucleotide phosphate oxidase (Nox), which overproduces superoxide and H_2O_2 and creates Loop 3 as shown in Fig. 4. In particular, hydroxyl radicals seem to be in excess because of the absence of distinct biological scavengers. In the present study, H_2 seems to have disrupted this positive feedback loop by scavenging hydroxyl radicals. Levels of

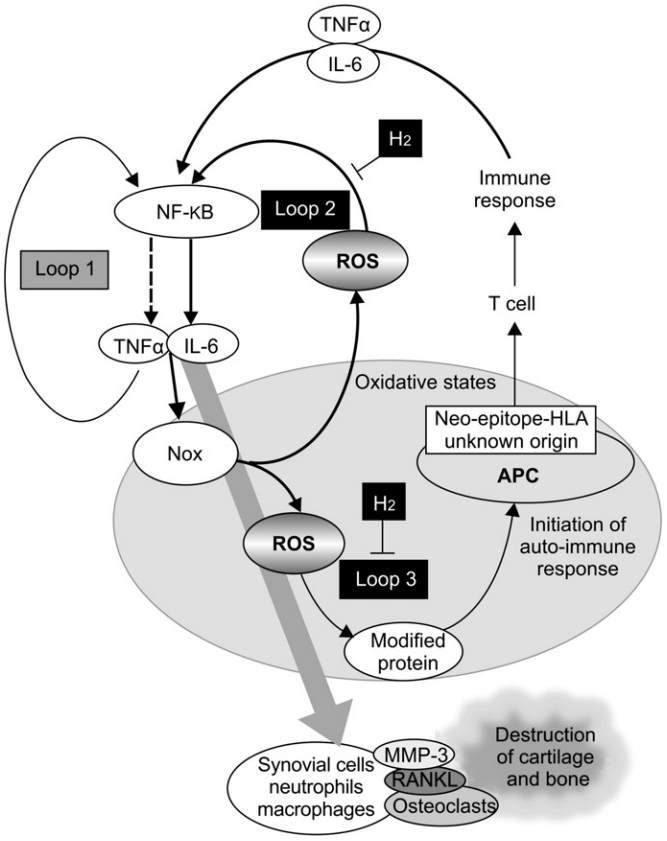

Fig. 4. A schematic representation of the 3 loops involved in amplification of inflammation in patients with rheumatoid arthritis. Loop 1 depicts the nuclear factor (NF)-κB-tumor necrosis factor-α (TNFα) positive feedback loop. Loop 2 depicts the redox sensing loop involving reactive oxygen species (ROS)-NF-κB-interleukin-6 (IL-6) and/or TNFα. In the present study, only Loop 2, which scavenges hydroxyl radicals directly or via NF-κB pathways, seemed to be blocked by H_2 treatment. ROS, which are generated by the Nox system and are amplified through these loops, then stimulate synovial fibroblasts, neutrophils, and macrophages that promote cartilage and bone erosion via matrix metalloproteinase (MMP)-3 or RANKL expression. In addition, the proteins modified by ROS may generate a Loop 3, which may promote an autoimmune response by feeding back into Loops 1 and 2.

IL-6, one of the factors responsible for the pathogenesis of RA, were reduced 4 weeks after the infusion of H_2, thereby corroborating the improvement in DAS28. On the other hand, another important cytokine, TNFα, which is also involved in the positive feedback loop, did not decrease. The mechanisms through which these 2 key cytokines participate in the pathogenesis of RA are different and often clinically complement each other [22]. Within the scope of the present study, H_2 may ameliorate disease activity through reducing IL-6-mediated inflammatory responses (see Loop 2 in Fig. 4). Although the transcription of IL-6 is under the influence of NF-κB, there may be an unknown mechanism which links IL-6 directly with oxidative stress or H_2 itself. It should be noted that the absence of TNFα reduction is also inconsistent with a previous study of experimental animal models in which H_2 was used as an anti-inflammatory electron donor, and TNFα was downregulated [9,23]. As it was reported with RA patients for whom MTX, the anchor drug for RA, was therapeutically effective [24], IL-6 may be more responsible for disease susceptibility than TNFα. However, the lack of influence of H_2 on TNFα may be explained by the duration of the study period, as the effect of H_2 on TNFα levels may have been missed. In addition, it should be noted that the disease duration of most patients in the present study was quite long (4 years and 7 months on average); therefore, the T cells responsible for the chronicity of RA were likely well established, and the disease duration may thus have influenced the effect of H_2 on TNFα. It is well-known that there are important windows of opportunity for establishing therapeutic efficacy of TNFα inhibitors and conventional DMARDs [25]. It should be noted that the number of patients in this study is very small, and the outcome to be concluded is limited. Thus, in order to investigate the mechanism of action of H_2, large-scale clinical investigations of early RA patients are necessary.

The prognosis and therapeutic responses in RA are influenced by the presence of ACPA [1,26,27]. In the present study, we also observed a correlation between therapeutic efficacy of infused H_2 and ACPA. The infusion of H_2 was less effective in the ACPA-positive patients. However, the total dose of H_2 and the treatment period was restricted here. It is important to investigate the efficacy of infused H_2 with more frequent and longer treatments. In addition, the change in the value of ACPA after treatment with infused H_2 should be observed over a longer period.

It is currently difficult to identify the difference between passive diffusion of H_2 from the stomach and the diffusion of H_2 from circulating venous flow. However, in the present study, all patients who were administered H_2-saline exhibited a decrease in DAS28 on days 6–8; the amount of H_2 molecules infused was below 0.5 mg a day, which is less than the amount of H_2 in high H_2 water (>2 mg). Although the total amount of H_2 administered during the 5-day treatment was less than 2.5 mg, the influence of infused H_2-saline on improving inflammation was comparable to that observed with high H_2 water (total 56 mg over 4 weeks). The absence of the significant decrease of the urinary 8-OHdG immediately post-infusion may be due to the small amount of H_2 taken in the venous flow, although it seems to be sufficient to disrupt the ROS-cytokine amplification circle shown in Fig. 4. However, further study is necessary to evaluate the additional benefits of this administration method.

The reason why such a small amount of H_2 has proven effective in patients with active RA may be explained by circulating blood flow. It is well-known that the whole blood (approximately 5–6 l) makes a round of the circulatory system in approximately 1 min in an adult weighing 50 kg. In the present study, 500 ml of H_2-saline was drop infused for 40 min. This indicates that even though the concentration of H_2 in the venous flow was quite low [28], the circulating immune cells, including lymphocytes, neutrophils, monocytes, and macrophages, which are responsible for the chronic inflammation of the synovium, are circulated and exposed to at least 40 times the highest concentration of H_2 in the venous flow during treatment. While it is still unclear how many H_2 molecules were absorbed in the activated synovium in the inflamed joints, the influences observed in the present study are thought to be due to the anti-inflammatory action of H_2 molecules on the circulating immune cells. This hypothesis may explain the therapeutic efficacy of the infused H_2, in spite of the absence of decreased 8-OHdG levels immediately post-infusion, which is thought to reflect the decrease in oxidative stress in the whole body. The significant reduction in urinary 8-OHdG levels in 4 weeks post-infusion may represent the improvement of the disease condition, which could cause an overall decrease in oxidative stress.

While Galen in ancient Greece first described the physiopathology of RA [29], effective RA therapy has emerged only recently. Glucocorticoid steroid hormone, which was first used for RA by Hench in 1949, is probably the first drug that dramatically improved the symptoms of RA [30]. However, it has been shown that glucocorticoids do not terminate the progression of RA, nor cure the disease. Beginning in the 1970s, DMARDs, including gold salts, were used for the early aggressive treatment of RA. Among the many DMARDs developed since then, low dose MTX, which was developed in the 1980s, has become an anchor drug used to control the disease activity of RA [31–33]. More recently, monoclonal anti-TNFα antibodies emerged in the 1990s as a potential RA therapy [34], and since then, the IL-6 inhibitor, tocilizumab, was approved in 2008 in Japan [3]. These progresses made a so-called paradigm shift, and disease remission has been achieved; however, it is still difficult to make patients completely drug-free and completely cure the disease [1]. One of the difficulties is that almost all of these therapies are based on immune-suppressive strategies and do not directly address the currently unknown disease origin. The application of H_2 described

here is quite different from those conventional immunosuppressive approaches. The anti-inflammatory properties of H_2 are thought to be based on the elimination of surplus, toxic free-radicals, including hydroxyl radicals and peroxynitrite. Therefore, the improvement in the disease activity of RA observed in this study seems to suggest that the pathophysiological disorder and the vicious circle of inflammation in RA are in part caused by excess radicals. These non-specific but effective anti-inflammatory capabilities of H_2 can affect undesired inflammation inherent in other diseases, including atherosclerosis, which also exhibits ROS-related inflammation pathways [5]. As the safety of H_2 has been established, it is possible to consider daily intake of H_2 [10,16]. This may alter the morbidity risk as well as the natural course of RA and related diseases. However, more intensive studies including a wide range of RA patients are necessary, not only to establish the best way of monotherapy using H_2, including the combination of drinking high H_2 water and venous infusion of H_2, but also to develop combination therapies with conventional DMARDs, biological agents, or glucocorticoids. Furthermore, the efforts to investigate the unknown bioactivities of H_2 should be continued, as there remain unexplainable factors in the therapeutic efficacy of H_2 [5,10].

In conclusion, we have demonstrated that intravenous infusion of H_2 is effective in treating RA. This method could be useful not only for controlling RA but also for preventing age-related inflammatory diseases.

Authors' contributions

T. Ishibashi designed the study and analyzed all of the data. In addition, he formulated and tested the hypotheses and derived conclusions. B. Sato provided the instrument for preparing the H_2-saline. S. Shibata helped in data collection. T. Sakai performed the statistical analyses. Y. Hara, Y. Naritomi, S. Koyanagi, and H. Hara supported this study by offering space, collecting data, and giving advice. T. Nagao helped in designing this study and gave advice on many aspects.

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