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Original Article

Molecular hydrogen decelerates rheumatoid arthritis progression through inhibition of oxidative stress

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Abstract: Rheumatoid arthritis (RA) is a chronic inflammatory disease which results in progressive destruction of the joint. In this study, we examined if the hydrogen could inhibit inflammation in a mouse model of collagen-induced arthritis (CIA) via oxidative stress on RA-FLSs. Moreover, to identify the mechanisms of action, we evaluated the effect of hydrogen on RA-FLSs development and the expression of pro-inflammatory cytokines and signaling pathways. Based on our result, H_2 enriched medium can increase super oxide dismutase (SOD) level following H_2O_2 treatment and decrease 8-hydroxy-2'-deoxyguanosine (8-OHdG) level. Since H_2O_2 treatment activates MAPK, NF-κB and TGF-β1 in cells, our study suggested that H_2 could inhibit H_2O_2 activated MAPK and NF-κB activation as well as TGF-β1 expression in treated cells. Taken together, our data suggested that H_2 can directly neutralize OH and $ONOO^-$ to reduce oxidative stress. Moreover, MAPK and NF-κB pathway also play roles in oxidative damage caused by H_2O_2 in RA-FLSs. H_2 can provide protection to cells against inflammation, which may be related to inhibition of the activation of MAPK and NF-κB.

Keywords: Rheumatoid arthritis, hydrogen, SOD, NF-κB, TGF-β1

Introduction

Rheumatoid Arthritis (RA) is an autoimmune disease that usually occurs between 30-55 years of age with a female preponderance [1]. RA is characterized by irreversible joint damage accompanied by destruction of bone and cartilage [1]. The pathophysiology of RA comprises of several events initially characterized by infiltration of neutrophils, lymphocytes and monocytes which results in severe inflammation. Previous reports suggested that oxidative stress (OS) plays an important role in the pathogenesis of RA [2]. Antioxidants and anti-oxidative enzymes have been shown to reduce cartilage damage in animal models of RA [3, 4].

Generally, OS results from the various oxidizing species such as reactive oxygen species (ROS) and could be defined as an imbalance between oxidants and antioxidants, which leads to a disruption of redox signaling and control or molecular damage [5]. There are two main families of oxidants, which include reactive oxygen species (ROS) and the reactive nitrogen species (RNS) [6]. It has been reported that the synovial fluid and peripheral blood of RA patients have high levels of ROS and ROS-generated molecules, including superoxide, peroxide, hydroxyl radicals as well as RNS such as peroxynitrite [7, 8].

Mitogen-activated protein kinases (MAPK) signaling pathway is closely related to cell proliferation and apoptosis which could be stimulated by oxidative stress, hormone or cytokine activation. In RA, OS can activate MAPK by affecting the proliferation of fibroblasts [9]. But the changes of MAPK in RA are unknown. It has been reported that high levels of TGF-β are present in synovial fluid of RA patients, which may be partially responsible for the pathologic changes observed in the synovial lining layer [10].

Hydrogen is one of nature's molecules and recent research has demonstrated that inhaling hydrogen, or consumption of hydrogen-rich water played a protective role in heart, brain and liver ischemia-reperfusion injury by neutralizing OH and $ONOO^-$, in vitro [11]. As a new selective free-radical material, hydrogen may be particularly suitable for the treatment of RA induced oxidative stress [10, 12, 13].

In this study, we hypothesized that hydrogen would decrease inflammatory responses, in a mouse model of collagen-induced arthritis (CIA), by subduing oxidative stress on RA-FLSs. To identify the mechanism of action, we studied the influence of hydrogen on RA-FLSs development, expression of pro-inflammatory cytokines and signaling pathways.

Material and methods

Animals

Male DBA/1J mice aged 8~10 weeks were purchased from Shanghai Institute of Medical Material, Chinese Academy of Sciences (Shanghai, China). All animals were housed under specific pathogen-free conditions for 2 weeks prior to commencement of experiments. The experiment were performed on animals aged 7~10 weeks. All experimental procedures were evaluated and approved by the Animal Research Ethics Committee of the Jinling hospital.

Hydrogen-saturated medium and hydrogen-saturated saline preparation

The hydrogen-saturated medium and hydrogen-saturated saturated saline were prepared as previously described [14]. Briefly, molecular hydrogen was dissolved in Dulbecco's modified Eagle's medium (DMEM) or saline for 2 h under high pressure (0.4 MPa) to a supersaturated level using the hydrogen-rich-water-producing apparatus (Blue Mercury Inc., Tokyo, Japan). The hydrogen saturated medium or saline was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume. Hydrogen-rich water was prepared fresh to ensure that the hydrogen concentration was more than 0.6 mM as measured by a hydrogen sensor (Unisense, Denmark).

Induction and evaluation of CIA

For the CIA model, 100 μg of bovine type II collagen (Chondrex Inc., Redmond, WA) was emulsified with an equivalent volume of Freund's complete adjuvant (Sigma, St. Louis, MO) and injected intradermally at the base of the tail into DBA/1J mice [15]. Following a similar protocol, adjuvant-treated littermates that were given phosphate buffered saline (PBS) instead of type II collagen served as control (n=10). The CIA model DBA/1J mice were randomly divided into two groups (n=10 each group): control group (saline treated), hydrogen-saturated saline-treated group (10 ml/kg/day). Commencing on day 7 after primary immunization, hydrogen-saturated saline was intraperitoneally injected once daily. Mice were observed from day 21 post-immunization for scoring and clinical symptoms. Four-five days after the implantation, the mice were killed [16].

To evaluate the effects of hydrogen on CIA development and progression, DBA/1J mice were employed. Oral hydrogen (10 ml/kg/day) was administered for 45 days, commencing 7 days after primary immunization.

Histopathological analysis

Ankle joints of mice were removed, fixed in 4% (v/v) paraformaldehyde, decalcified, embedded in paraffin, and sectioned (5 mm). The sections were stained with hematoxylin-eosin (H&E).

Cell culture

Rheumatoid arthritis fibroblast-like human synovial cell line RA-FLSs was purchased from Jennio Biotech Co., Ltd. (Guangzhou, China). The cells were cultured in DMEM with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2 atmosphere. The medium was changed every 3 days and were used between the 5^{th}~10^{th} passages.

Cell proliferation assay

Cell proliferation was assayed using MTT assay. In brief, cells were seeded at a 1×10^5/ml concentration in 100 μl culture medium in a 96-well plate. The cells were treated with 0.8 mM hydrogen peroxide (H_2O_2) and co-treated with 0.6 mM hydrogen for 24 h and same volume of serum-free DMEM served as negative control. 20 μl MTT solution was added to every well and incubated for 4 hours. The culture medium was removed, 150 μl dimethyl sulphoxide was added to solubilize the MTT formazan salt and the absorbance of solution was measured at 570 nm using a micorplate reader (Bio-Rad, Hercules, CA, USA).

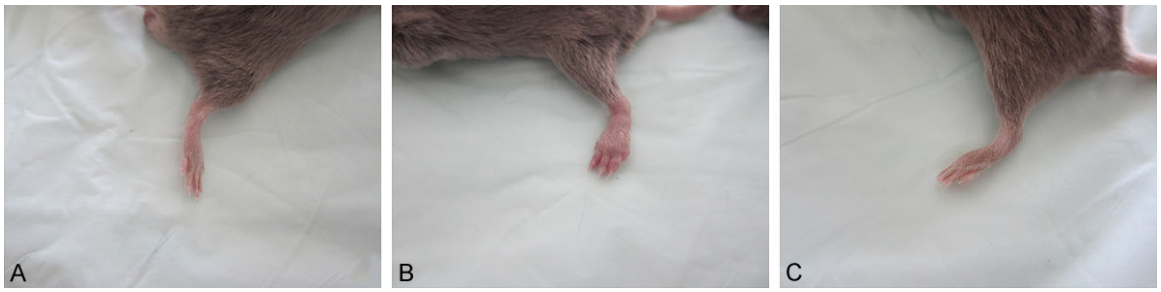

Figure 1. Pictographs showing gross observation of animal joints in mouse model of RA. A. Normal animal. B. Joint swelling in CIA mice model. C. Joint swelling reduced in hydrogen-treated mice.

Assays of cellular SOD and GSH concentrations

Oxidative damage is prevented by the rapid scavenging of O_2^- by the mitochondrial enzyme manganese superoxide dismutase (SOD). Glutathione (GSH) is a powerful intracellular antioxidant and plays a role in the detoxification of a variety of electrophilic compounds. Cells were seeded in 24-well plates (2×10^4 cells/well) and subjected to the treatment (H_2O_2 and co-treated with 0.6 mM hydrogen) for 24 h. Then, the supernatants were collected and assayed for SOD and GSH, following the manufacturer's instructions (Jiancheng Bioengineering Co., Nanjing, China).

Enzyme-linked immunosorbent assay (ELISA)

RA-FLSs were seeded in 24-well plates and treated (H_2O_2 and co-treated with 0.6 mM hydrogen) for 24 h. Then, the treated cells were lysed with a cell lysis buffer and the supernatant was collected for the estimation of cytokines, such as 8-OHdG, MAPK, NF-κB and TGF-β1 using commercially available human cytokine ELISA assay kits, according to the manufacturer's protocol (Jiancheng Bioengineering Co., Nanjing, China).

Statistical analysis

All data are presented as means ± SD. Differences in indicators between treatment samples or groups, such as cytokines levels between the different groups were assessed by Student *t*-test. A two-tailed *p*-value less than 0.05 was considered significant.

Results

Hydrogen attenuates the development and progression of arthritis in CIA mice

In the CIA model group, joint swelling was observed compared to control (**Figure 1A** and **1B**). Moreover, in the histopathological examination, evidence of arthritis such as synovial hyperplasia, arthrostenosis, inflammatory cell infiltration and cartilage destruction was observed (**Figure 2B**). Taken together, these data that suggested our model was successful. Recent studies have demonstrated that hydrogen may play a protective role in RA development by reducing oxidative stress [10, 12, 13]. Therefore, we expected an attenuation of severity of arthritis in CIA mice. As demonstrated in **Figures 1C** and **2C**, histopathological changes were less evident in hydrogen-treated mice compared to controls. The arthritis score and disease incidence in hydrogen-treated group was reduced (**Figures 1C** and **2C**). Therefore, our data suggests that hydrogen treatment demonstrated a protective role in the prognosis of RA.

Effects of hydrogen on cell proliferation

To further explore the mechanism behind the therapeutic effects of hydrogen on RA, we examined the proliferation rate using a modified MTT assay to see if hydrogen plays a role in cell proliferation. RA-FLSs were treated with H_2O_2, or H_2O_2 and hydrogen. Untreated synovial cells were included as control. The assay shows that the proliferation rate of cells co-treated with hydrogen and H_2O_2 was decreased compared with cells treated with H_2O_2 alone, which suggests that hydrogen reduced H_2O_2 stimulated cell proliferation in vitro (**Figure 3**).

Effects of hydrogen-rich medium on cellular oxidative products and anti-oxidation status

When further compared the H_2O_2 treatment, H_2 treatment increased SOD level and decreased GSH level (**Figure 4**), 8-OHdG levels decreased in the H_2 treated group following the treatment time (**Figure 4**). These results indicate that the

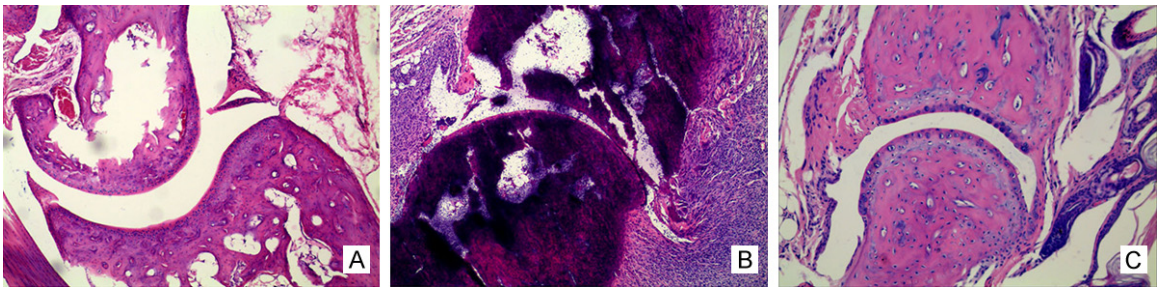

Figure 2. Histopathological changes in CIA mice and hydrogen-treated animals model (200×). Light micrographs of joint sections stained with hematoxylin and Eosin. A. There were no pathological changes in normal mice. B. Histopathological evidence of arthritis such as synovial hyperplasia, arthrostenosis, inflammatory cell infiltration and cartilage destruction was observed in CIA mice. C. Histopathological changes were less evident in hydrogen-treated than vehicle-treated mice.

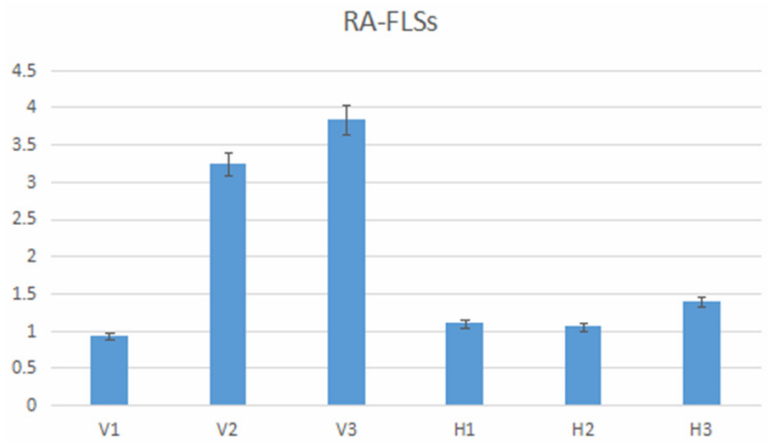

Figure 3. Cell proliferation in different groups. Histogram of the MTT assay shows that the proliferation rate of hydrogen and H_2O_2 was decreased compared with H_2O_2. With the extension of processing time, the H_2O_2 groups in the proliferating cells of the H_2O_2 and hydrogen group had no significant difference. (V represented H_2O_2 group, H represented H_2O_2 and hydrogen group, 1 represented 24 h, 2 represented 48 h and 3 represented 72 h).

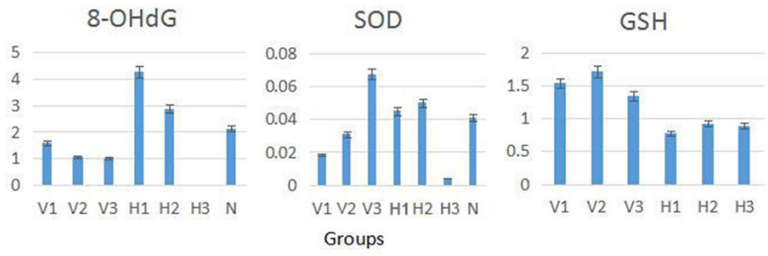

Figure 4. Effects of hydrogen-rich medium on cellular oxidative products and antioxidant status. Sample histographs comparing the H_2O_2 treatment, H_2 treatment increased SOD level and decreased GSH level. 8-OHdG levels decreased in H_2 treatment group following treatment time. (V represents H_2O_2 group, H represents H_2O_2 and hydrogen group, 1 represents 24 h, 2 represents 48 h, 3 represents 72 h and N represents normal control).

hydrogen molecule had significant effect on oxidative stress.

Effects of treatment with hydrogen on MAPKs and NF-κB pathway

NF-κB transcription family plays an important role in the inflammatory response of RA progression. MAPKs extensively regulate RA-related events, especially in the expressions of proteases. As predicted, exposure of H_2 decreased significantly the MAPKs and NF-κB activation in RA-FLSs (**Figure 5**). These results indicate that H_2 might inhibit the inflammation process in H_2O_2-treated RA-FLSs by regulating the intracellular MAPK and NF-κB pathways.

Effects of treatment with hydrogen on TGF-β1 expression

TGF-β1 regulates cell growth, adhesion and differentiation in a variety of cell types. We assessed the effect of H_2 on TGF-β1 activation in synovial cells. Pre-incubation of RA-FLSs with H_2O_2 elevated TGF-β1 expression within the cytosol and decreased TGF-β1 expression with H_2. The H_2O_2 effect was significantly reversed by H_2 (**Figure 6**).

Discussion

In this paper, we showed that H_2 exerted therapeutic effects in a mouse model of RA and in

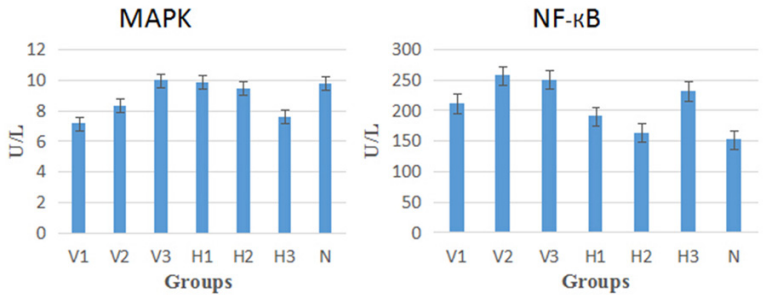

Figure 5. Effects of hydrogen treatment on MAPKs and NF-κB pathway. Representative histographs showing that exposure to H_2 decreased MAPKs and NF-κB activation in RA-FLSs significantly, compared with other groups. (V represents H_2O_2 group, H represents H_2O_2 and hydrogen group, 1 represents 24 h, 2 represents 48 h, 3 represents 72 h and N represents normal control).

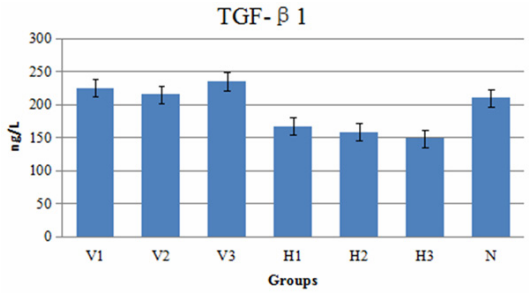

Figure 6. Pre-incubation of RA-FLSs with H_2O_2 elevated TGF-β1 expression within the cytosol and decreased TGF-β1 expression with H_2. Histogram showing the effect of H_2O_2 was significantly reversed by H_2. (V represents H_2O_2 group, H represents H_2O_2 and hydrogen group, 1 represents 24 h, 2 represents 48 h, 3 represents 72 h and N represents control).

human RA-FLS cells. The probable mechanisms of action of H_2 were also further elucidated. Our data suggested that H_2 could reduce the levels of oxidative products and attenuate H_2O_2 induced over proliferation in RA-FLSs.

Many studies have demonstrated a role of oxidative stress in initiation and progression of chronic inflammatory disease such as RA [9]. Recently, it has been demonstrated that consumption of water with a concentration of molecular hydrogen significantly improved the disease activity and reduces the oxidative stress in RA, which may imply a novel therapeutic target in RA [7, 12].

Rheumatoid arthritis involving a fibrous synovial cell (FLS) led to the elevation of oxidative stress levels with a corresponding to weakening of antioxidant capacity. SOD in antioxidant system could eliminate O_2^-, therefore playing a significant role in maintaining the dynamic balance of ROS. In our study, we found that H_2 medium can increase SOD levels following H_2O_2 treatment with a decrease in 8-OHdG level at the same time, which implied that H_2 can directly neutralize OH and $ONOO^-$.

Rheumatoid synovial cells are involved in variety signaling pathways. MAPK pathway activated NF-κB regulates cell growth, proliferation, differentiation, apoptosis and other physiological processes. A large amount of gene expression during inflammatory response is regulated by NF-κB such as TGF-β1, a key cytokine regulating cell growth and differentiation. The expression of TGF-β1 is very low in normal synovial joints and could be upregulated during the RA [17]. Our data suggested that H_2 treatment reduced the expression of MAPK, NF-κB and TGF-β1 in H_2O_2 treated RA-FLSs. These results indicated that MAPK, NF-κB pathway played a role in oxidative damage caused by H_2O_2 in RA-FLSs.

Our results showed that H_2 inhibited oxidative stress levels in a RA model and cells. H_2 reduced the levels of oxidative stress in RA-FLSs and reduced the abnormal proliferation. These results may be associated with the inhibition of the activity of MAPK and NF-κB. However, many factors could induce the cells. In conclusion, our data demonstrated that H_2 could be a novel therapeutic molecule in the treatment of RA and further investigation is need to explore its exact role.

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Disclosure of conflict of interest

None.

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