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, H2 enriched medium can increase super oxide dismutase (SOD) level following H2O2 treatment and decrease 8-hydroxy-2′-deoxyguanosine (8-OHdG) level. Since H2O2 treatment activates MAPK, NF-κB and TGF-β1 in cells, our study suggested that H2 could inhibit H2O2 activated MAPK and NF-κB activation as well as TGF-β1 expression in treated cells. Taken together, our data suggested that H2 can directly neutralize OH and ONOO- to reduce oxidative stress. Moreover, MAPK and NF-κB pathway also play roles in oxidative damage caused by H2O2 in RA-FLSs. H2 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].
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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% CO2 atmosphere. The medium was changed every 3 days and were used between the 5th~10th 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 (H2O2) 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 sulfoxide 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).
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Assays of cellular SOD and GSH concentrations

Oxidative damage is prevented by the rapid scavenging of $\text{O}_2^-$ 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 (2×10⁴ cells/well) and subjected to the treatment ($\text{H}_2\text{O}_2$ 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 ($\text{H}_2\text{O}_2$ 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, arthrosclerosis, 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 $\text{H}_2\text{O}_2$, or $\text{H}_2\text{O}_2$ and hydrogen. Untreated synovial cells were included as control. The assay shows that the proliferation rate of cells co-treated with hydrogen and $\text{H}_2\text{O}_2$ was decreased compared with cells treated with $\text{H}_2\text{O}_2$ alone, which suggests that hydrogen reduced $\text{H}_2\text{O}_2$ stimulated cell proliferation in vitro (Figure 3).

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

When further compared the $\text{H}_2\text{O}_2$ treatment, $\text{H}_2$ treatment increased SOD level and decreased GSH level (Figure 4), 8-OHdG levels decreased in the $\text{H}_2$ treated group following the treatment time (Figure 4). These results indicate that the
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In this paper, we showed that H₂ exerted therapeutic effects in a mouse model of RA and in...
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human RA-FLS cells. The probable mechanisms of action of $\text{H}_2$ were also further elucidated. Our data suggested that $\text{H}_2$ could reduce the levels of oxidative products and attenuate $\text{H}_2\text{O}_2$ 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 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 $\text{H}_2$ treatment reduced the expression of MAPK, NF-κB and TGF-β1 in $\text{H}_2\text{O}_2$ treated RA-FLSs. These results indicated that MAPK, NF-κB pathway played a role in oxidative damage caused by $\text{H}_2\text{O}_2$ in RA-FLSs.

Our results showed that $\text{H}_2$ inhibited oxidative stress levels in a RA model and cells. $\text{H}_2$ 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 $\text{H}_2$ 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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