Molecular Hydrogen: New Antioxidant and Anti-inflammatory Therapy for Rheumatoid Arthritis and Related Diseases

Toru Ishibashi*

Haradoi Hospital, Department of Rheumatology and Orthopaedic Surgery, 6-40-8 Aoba, Higashi-ku, Fukuoka 813-8588, Japan

Abstract: Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects approximately 1% of the population. It is characterized by irreversible joint disorder accompanied by destruction of bone and cartilage, which causes serious morbidity. In addition, the chronic inflammation associated with RA can increase one’s risk of atherosclerosis, which is a significant cause of mortality with the cardiovascular failure [1, 2]. Atherosclerosis associated with RA progresses rapidly, even in the absence of the conventional risk factors, such as hypertension, diabetes mellitus, or obesity. Consequently, the aim of RA therapy includes not only improving the disease activity, which is commonly estimated by the joint disorder activity index (DAS28), but also controlling the systemic and unremarkable inflammation of endothelial cells. Recent progress of anti-cytokine therapies is improving the risk for cardiovascular disease (CVD) [3].

Although the etiology is unknown, RA is certainly associated with autoimmune disorders, and its pathogenesis has been well investigated [2]. Auto-reactive T cells that infiltrate the synovial tissue promote the immune response, resulting in an overproduction of pro-inflammatory cytokines such as tissue necrosis factor alpha (TNFα), interleukin 1 (IL-1) and interleukin 6 (IL-6). Accordingly, early therapy was based on aggressive biological modification of the disease by controlling the synovial T cells and/or reducing the levels of the cytokines. Unfortunately, this approach has met limited therapeutic success, raising the issue that important regulatory factors were missing in the existing mechanistic model of RA. Reactive oxygen species (ROS) could be one of the unidentified regulatory factors. The synovial fluid and peripheral blood of RA patients have high levels of ROS and ROS-generated molecules, including superoxide, peroxide, hydroxyl radicals and reactive nitrogen species like peroxynitrite [3-4]. They oxidize various cellular and extracellular components, including nucleotides, DNA, proteins, polysaccharides, and lipids, by means of their unpaired free radicals. Of these, 8-hydroxyguanine (8-OHdG), which is produced by the oxidation of guanine bases in DNA and also in the nucleotide pools, is considered important [7-9]. 8-OHdG is a standard biomarker for oxidative stress. Numerous studies have reported that 8-OHdG accumulates in diseases related to oxidative stress, such as cancer, diabetes mellitus, Alzheimer’s disease, hypertension, cardiovascular disease, metabolic syndrome, and autoimmune disease [10-16]. Elevated levels of 8-OHdG have been reported in RA [17, 18] and atherosclerosis [19].

In the past decade, it has been shown that molecular hydrogen (H₂) selectively eliminates highly reactive hydroxyl radical in cultured cells and living organisms [20, 21]. H₂ targets hydroxyl radicals, but not super oxide, peroxide, or nitric oxide, which are important molecules for organisms [22]. Recently, it was demonstrated that consumption of water with a high concentration of molecular hydrogen (4-5 ppm in the water) significantly improves the disease activity and reduces the oxidative stress in RA [23]. H₂ seemed to complement or provide a substitute for conventional therapy by reducing oxidative stress and improving damage associated with RA, especially in the early stages of the disease and in the case of Antibodies against cyclic citrullinated peptide (ACPA)-negative RA.

In this review article, prospective applications of new H₂ therapies, for both the diagnosis and treatment of RA, are discussed. Also the possible expectation for the prevention of RA and related atherosclerosis by the daily consumption of high H₂ water are mentioned.

Keywords: Rheumatoid, atherosclerosis, prevention, Oxidative Stress, 5 ppm, Molecular Hydrogen, 8-hydroxyguanine, Hydroxyl Radical.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects approximately 1% of the population. It is characterized by irreversible joint disorder accompanied by destruction of bone and cartilage, which causes serious morbidity. In addition, the chronic inflammation associated with RA can increase one’s risk of atherosclerosis, which is a significant cause of mortality with the cardiovascular failure [1, 2]. Atherosclerosis associated with RA progresses rapidly, even in the absence of the conventional risk factors, such as hypertension, diabetes mellitus, or obesity. Consequently, the aim of RA therapy includes not only improving the disease activity, which is commonly estimated by the joint disorder activity index (DAS28), but also controlling the systemic and unremarkable inflammation of endothelial cells. Recent progress of anti-cytokine therapies is improving the risk for cardiovascular disease (CVD) [3].

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In this review article, prospective applications of new H₂ therapies, for both the diagnosis and treatment of RA, are discussed. Also the possible expectation for the prevention of RA and related atherosclerosis by the daily consumption of high H₂ water are mentioned.

GENERATION OF ROS IN CHRONIC INFLAMMATION

ROS are produced as an inevitable byproduct of electron transfer in oxidative phosphorylation during aerobic metabolism [24]. On the other hand, during inflammatory stages of RA, infiltration or proliferation of immune activated cells in the synovium actively generate ROS via the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase system (Nox) [25,26,27]. Among the actively generated ROS, superoxide anion is the primary product and liberated into extracellular matrix as well as sequestered in lysosomes. Su-
In addition to the oxidative stress, the NF-κB also stimulates the production of ROS and pro-inflammatory cytokines, both of which contribute to the accumulation of oxidative stress. In the synovial tissue, high levels of the pro-inflammatory cytokines activate and recruit neutrophils, synovial fibroblasts, and macrophages, which contribute to further overproduction of ROS by the Nox system. This ROS re-enters the pathway, upstream of NF-κB, creating another positive feedback loop within the synovial inflammation pathway (see the "Loop 2" in Fig. 1). These loops stimulate the production of ROS and pro-inflammatory cytokines, both of which contribute to the accumulation of oxidative stress.

In addition to the oxidative stress, the NF-κB also stimulates proteolytic activity. Proteolytic enzymes play the central role in the destruction of cartilage and bone associated with RA. Cytokines including TNFα or IL-1 activate synovial fibroblasts and macrophages to produce cartilage-destroying enzymes, such as metalloproteinases (MMP), including MMP-1 and MMP-3. They are thought to be secreted from the synovial fibroblasts and macrophages especially in the pannus of advanced rheumatoid joints and can degrade hyaluronic acid, proteoglycan, and collagen which construct cartilage [40]. Also, chondrocytes stimulated by IL-1 secrete MMP-3 and promote the absorption of the subchondral bones [41, 42]. MMP-3 is considered an important protease in RA joint destruction as the concentration of MMP-3 in the serum of RA patients is correlated with disease activity [43]. In addition, the activation of TNFα leads to the elevated expression of molecules, such as RANKL (receptor activator of NF-κB ligand), related to bone-destruction [44]. Expression of RANKL in the synovial fibroblasts results in differentiation of the osteoclast progenitor cells expressing RANK on their surface. The progenitor cells, including monocytes, which infiltrate in the synovial tissue, mature into osteoclasts—the primary cells responsible for the absorption of bone—which are responsible for the bone erosion associated with RA [2, 45]. Again, ROS located upstream of these cytokines, are indirectly responsible for the destruction of cartilage and bone in the joints of RA patients (Fig. 1). It has also been discussed that the synovial fluid in RA is hypoxic and the synovial reperfusion seems to stimulate the redox cascade including NF-κB [36].

ROLE OF ROS IN REDOX REGULATION OF NF-κB-DEPENDENT INFLAMMATORY CASCADES IN RA

Oxidative stress regulates several cellular processes, including the signaling pathways responsible for inflammatory responses. The activation status of signaling intermediates is regulated by ROS/GSH-mediated modifications of their thiol (R-SH) groups [33]. Excess ROS production disrupts this redox balance and amplifies inflammatory responses via NF-κB. This leading transcription factor induces the transcription of several pro-inflammatory cytokines. All of the ROS described above cause an imbalance of redox state within the inflamed tissue, resulting in the activation of NF-κB and related transcription factors. NF-κB is a central regulator of cellular inflammatory response as it controls many of the genes involved in inflammation [35, 36]. It induces the transcription of several pro-inflammatory cytokines including TNFα, IL-1, and IL-6, which play key roles in the progression of RA and therefore are therapeutic drug targets. Among these, TNFα plays a pivotal role in the persistent inflammation of RA synovium, through its stimulation of oxidative stress [36, 37]. TNFα, produced downstream of the NF-κB transcriptional complex, re-activates NF-κB by releasing it from IκB, which in steady state phosphorylates and inactivates NF-κB by forming complex with this transcription factor [38, 39]. In this manner, the “Loop 1” acts as a positive feedback mechanism within the NF-κB and TNFα system and promotes the inflammatory response (see the “Loop 1” in Fig. 1).

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Recent successful anti-inflammatory therapies targeting TNFα seem to be attributed to disrupting the inflammatory Loop 1. These findings suggest that a therapeutic approach targeting the ROS-TNFα amplification loop would significantly reduce inflammation-mediated damage in the joints of RA patients. As evident in (Fig. 1), such a therapy that breaks down the ROS feedback loop may be effective and necessary for preventing damage associated with RA.

DIRECT DAMAGE OF JOINT TISSUE BY ROS

Proteolytic activity is not the only source of cartilage and bone destruction associated with RA. ROS also have direct effects as they oxidize and degrade the major components of cartilage and bone, including collagen and hyaluronic acid (HA) [6]. Numerous studies have detected products of ROS reactivity in serum as well as in the joint fluids from patients with RA [5]. The presence of hyaluronic acid in reduced state and abundant leukocyte infiltration are common features of RA joint fluid. The degradation of HA is, at least in part, the result of fragmentation by hydroxyl radical [6, 46, 47]. The decreased viscosity of joint fluid in RA patients is thought to be attributed to such degradation of HA. Hydroxyl radicals also modify collagen, resulting in the formation of cross-links or fragmentation of the protein [48, 49]. Such degraded collagen seems to be more susceptible to subsequent cleavage by proteinase in the joint fluid.

These degrading processes and subsequent tissue remodeling of the synovial tissue could invite phagocytic and proteinase activities, which result in the induction of ROS-cytokine activated inflammatory ‘Loop 1 and Loop 2’, as shown in (Fig. 1). The positive feedback resulting from the accumulation of oxidized products could further exacerbate the cartilage and bone erosion, and lead to joint destruction. Therefore, protection of the joint components from the direct degradation by ROS represents another potential target of RA therapy.

GENETIC ASSOCIATION WITH RA AND ROLE OF ROS IN THE ETIOLOGY

Genetic modifications in the human leukocyte antigen (HLA) region account for nearly half of the genetically-related risk factors of RA. For instance, patients carrying the shared epitope (SE)—the region encoding QKRAA, QRRAA, or RRRAA amino acid sequences—in the third hyper-variable region of the human leukocyte antigen-DRB1 (HLA-DRB1) have a high susceptibility to develop RA [50, 51]. The risk for RA was reported as 15% for monozygotic twins carrying the SE [52], compared to 1% in the general population. This motif predicts not only the susceptibility to RA but also the severity of prognosis [51]. Recently it has been postulated that the combination of SE with other factors such as genetic polymorphisms and/or environment interact to increase the susceptibility to RA. First, it has been reported that significant positive interactions between HLA-DRB1-SE and smoking and the auto-antibody named ACPA incur the highest risk for the onset of RA [53, 54]. Second, functional mutations in the antioxidant enzyme GST are involved in increasing the genetic susceptibility of RA patients as they increase oxidative stress. The GST Mu-1 (GSTM1-null) genotype, which results in complete inhibition of the enzyme activity, exerts significant additive interactions with HLA-DRB1-SE on the risk of being ACPA-positive in RA patients [55]; this study provides evidence that oxidative stress increases susceptibility for the development of RA.

Oxidative stress may participate in the etiology of RA through direct interaction with DNA. For example, 8-OHdG is produced by the oxidation of guanine bases of DNA and is also present in the nucleotide pools [7-9]. It is highly mutagenic because it pairs with adenine as well as cytosine. This property causes transversion mutations during DNA replication. Consequently, the possibility exists that ROS may induce somatic mutations that result in altered protein function and/or immunogenicity and function as neo-epitopes [56-58]. These could continue to activate the immune system [58, 59]. Elevated levels of 8-OHdG have been reported in RA [17, 18]. However, somatic mutations of genomic DNA do not appear to be responsible for the auto-antigen in RA. If they were, one would expect homogeneous and stable epitopes in the pocket of the APC in the synovial tissue; however, these have never been detected even after extensive research.

Another possibility is that ROS primes the immune response through effects on mitochondrial DNA (mtDNA). A somatic cell has multiple copies of mitochondria, which have individual genome susceptible to mutations caused by ROS [60-62]. Somatic mutations of mtDNA have been reported to be associated with RA [63]. There is a slightly greater chance that the mutations of mtDNA are responsible for priming the immune response because it would be more difficult to demonstrate. Possible mutations in mtDNA and five mutated peptides that could potentially represent a SE have been identified in RA patients [64].

Regarding the potential role of ROS in RA etiology, more common considerations involve processes downstream from the DNA. For example, the mutagenic nucleic acid, 8-hydroxy-2'-deoxyguanosine, also causes partial phenotypic suppression during transcription [65]. It is likely that the error-proteins would be identified as foreign molecules and function as neo-epitopes [5, 58]. The error-proteins with alternative post-translational modifications are also included in this hypothesis. Antibodies for the post-translationally modified proteins containing citrulline are highly detected, especially among RA patients with poor prognosis. Although the relationship between ROS and loss of tolerance to citrullinated protein remains unclear, structural alterations of peptide motifs caused by oxidative modification may play a role. Because those antigens are oxidized post-translationally or post-translationally, they seem to be quite heterogeneous. If there is a possibility that such heterogeneous antigens could activate the immune response in a consistent manner, it seems that they must have a shared structure or electrochemically mutual characters, especially under the oxidative conditions. This hypothesis would establish a third loop, involving ROS, in the inflammation pathway (see Loop 3 in Fig. 1), involving priming of the auto-immune response to establish memory T cell clones against such neo-epitopes presented on antigen presenting cells (APC). In the state, down regulation of ROS seems to decrease the neo-epitopes of RA.

ATHEROSCLEROSIS ASSOCIATED WITH RA

Atherosclerosis is a common co-morbidity of RA, and a major cause of cardiovascular diseases (CVDs) and increased mortality among RA patients [66, 67]. While the pathogenesis of atherosclerosis usually involves the classical risk factors, including hypertension, obesity, or smoking, atherosclerosis associated with RA often occurs independent of these factors. Instead, the inflammatory cascades common to both RA and atherosclerosis, including pro-inflammatory cytokines and ROS appear responsible for the association between these disorders (see schematic Fig. 2). These inflammatory pathways affect the endothelial structure in blood vessels as well as synovial tissues in RA. Superoxide, produced by endothelial cells and smooth muscle cells via the Nox pathways including Nox1, Nox2, Nox4, and Nox5, are thought to be involved in endothelial dysfunction and the ROS-related progress of atherosclerosis [68-71]. In the case of atherosclerosis, oxidized LDL, located downstream of the intersection of these pathways, induces the formation of plaque responsible for the increased risk of CVDs [72, 73]. The development of atherosclerosis in RA patients is initiated by endothelial phenotypic alterations in response to large amounts of noxious stimuli. The increased expression of adhesion molecules such as intercellular adhesion molecules 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, the enhancement of pro-inflammatory cytokines (TNF-α, IL-1, interferon-γ), and the increase in oxidative stress initiate this condition
past decade, \( \text{H}_2 \) has been explored in the treatment of oxidative stress. Numerous studies in animals have shown the therapeutic potential of \( \text{H}_2 \) for diseases associated with ROS [22], and has already been used in some clinical treatment, such as type II diabetes mellitus, metabolic syndrome, hemodialysis, muscular diseases, and acute brain stem infarction [81-85]. The therapeutic potential of \( \text{H}_2 \) was first shown using the model for schistosomiasis-associated chronic liver inflammation. In the study, livers of schistosomiasis-infected mice were protected from the chronic inflammation by the treatment with 0.7 MPa hydrogen for two weeks [21]. Furthermore, it has also been shown that \( \text{H}_2 \) selectively eliminates the hydroxyl radical in cultured cells and living organisms [20]. In addition to its therapeutic potential for the elimination of hydroxyl radicals, \( \text{H}_2 \) is also used by deep-sea divers to prevent the decompression sickness, where its safety has been established [86, 87]. During these practical and experimental applications of \( \text{H}_2 \), no adverse effects have been reported.

Recently we reported that \( \text{H}_2 \) is the effective molecule not only for compensational use along with insufficient anti-rheumatic drugs but also for the diagnostic or monotherapeutic use at early stage in patients with RA [23]. In the study, patients were administered water containing 4-5 ppm \( \text{H}_2 \) (high \( \text{H}_2 \) water). By drinking 500 ml of the high \( \text{H}_2 \) water daily for four weeks, oxidative stress was effectively reduced and the disease activity in RA was significantly improved. \( \text{H}_2 \) seemed to have complemented conventional RA therapy by reducing oxidative stress. At least in the early stage of disease progression and among ACPA-negative patients, \( \text{H}_2 \) seems to have a potential to assist with diagnosis and treatment of non-aggressive or transient RA. Also considering the crossing mechanisms shown in (Fig. 2), we expect that the development of atherosclerosis in RA patients could be slowed or even prevented by the daily uptake of \( \text{H}_2 \) (shown in Fig. 2).

Many questions regarding the effectiveness of \( \text{H}_2 \) for the treatment of RA still remain. Among the concerns expressed by experts in the field are the short residence time and the extremely low amount of \( \text{H}_2 \) administered by drinking water with 1.6 ppm \( \text{H}_2 \) at the highest than the inhaled 2% \( \text{H}_2 \) gas. This discrepancy of more than 100 times of difference between them, even if they seems to have similar biological effects on organisms, have been discussed previously [80]. The authors proposed the possibility of \( \text{H}_2 \) for a gaseous signal modulator to explain the unknown mechanisms for \( \text{H}_2 \) effects. Another concern is related to alterations of gene expression that have been measured in response to \( \text{H}_2 \) using DNA microarray analysis [88]. In this study, \( \text{H}_2 \) caused the up-regulation of 548 and down-regulation of 695 genes in rat liver. Among these, the up-regulation of redox-related genes was especially prominent. The results raised the possibility that, while minimal in magnitude, numerous effects on the gene expression caused by \( \text{H}_2 \) may shift indirect molecular reactions in an organism. Furthermore, additional mechanisms related to the products of hydroxyl radical are still unknown. I discuss some of them in the next section.

**EXPECTATIONS REGARDING THE USE OF \( \text{H}_2 \) FOR THE PREVENTION OF RA AND Atherosclerosis**

In our study, \( \text{H}_2 \) seems to have an influence even during the washout period when the patients did not drink high \( \text{H}_2 \) water for 4 weeks [23]. The persistence of this effect cannot be explained by continuous elimination of hydroxyl radical. It is believed that \( \text{H}_2 \) reach into cytosol, nucleus and even into mitochondria of various tissues by gaseous diffusion [22]. \( \text{H}_2 \) administered via high \( \text{H}_2 \) water as well as \( \text{H}_2 \)-saturated water (containing 1.6ppm \( \text{H}_2 \)) rapidly reached the maximum concentration in the lung within 10 min and was exhaled within 60 min [23]. This indicates that the surplus \( \text{H}_2 \) passed through the body within a small period, except for the \( \text{H}_2 \), which seems to have reacted with hydroxyl radical. The removal of hydroxyl radicals and interference with the inflammatory response pathways described above likely resulted from exposure to \( \text{H}_2 \); however, other explanations are required to explain the continuation
of benefits observed once H2 administration had been terminated. The effects by hydroxyl radicals are rapid and detrimental, but the target molecules oxidized by hydroxyl radicals may carry the ‘reactive’ signal until it is degrades. Constitutive stimulation by such oxidized molecules may be eligible for the chronic inflammation of RA. One of such molecules is 8-OHdG, but other molecules that have been unknown or neglected should be investigated in the future.

By using H2 therapeutically for RA, some questions regarding the etiology of RA may be resolved. If modified antigen is produced by hydroxyl radical and the immunogenic molecules with the shared epitope are responsible for the onset of RA, H2 could eliminate the immunogenic state during the early stages of RA, resulting in only transient inflammation. In such cases, constitutive consumption of H2 would not be required, at least until the next trigger events such as infections or environmental stress occur. Alternatively, if the constitutive consumption of H2 would be required to improve RA even at the early stage, H2 seems to be effective except for the ‘Loop 3’ in (Fig. 1). Both of these schemas are possible. As it is widely believed that RA is divided into subclasses based on its mechanisms of onset or the prognosis, the efficacy of H2 in the treatment of RA may differ among the subclasses of RA.

Altogether, it is conceivable that H2 therapy has individual window of opportunity. The therapeutic window of opportunity is critical in determining the prognosis of RA, as usually observed with the conventional immunosuppressive drugs. If this window is missed, inflammation can advance and cause joint and bone destruction. The window for H2 therapy will depend on the stages where H2 works to reduce the inflammation, as shown in (Fig. 1). In cases where H2 therapy misses the opportunities, it may have only restricted effect on the inflammation of RA.

The recent success of and the paradigm shift within drug therapies seem to ignore the efforts to understand the origin of RA. When the precise relationship between RA and ROS is observed by H2, we have to remember the final goal to extirpate the auto-antigen responsible for RA.

Further study is required to confirm the therapeutic effectiveness of H2 on RA. Randomized, double blinded, and placebo-controlled investigation of the effects of high H2 water and the infusion of H2-resolved saline are ongoing (data not shown). We expect that H2 therapy in very early stage could prevent the onset of RA, if it is able to interfere with Loop 3 at the onset of the auto-immune response by reducing the ROS-related neo-epitope. Alternatively, the eventual activation of pro-inflammatory cytokines, which may trigger the auto-immune response, may be prevented by daily H2 consumption.

CONCLUSION

H2 is an inert gas present within the human body and is not classified as a medicine, but it has been shown to have therapeutic and diagnostic potential for RA as discussed here. The apparatus for creating H2-enriched water (over 5 ppm of H2) is already available commercially. The preventive effect of H2 may be shown by those populations who drink the high H2 water daily. Also, additional investigations of the benefits of H2 for RA patients are urgently needed, as H2 therapy has tremendous potential, but is currently under-utilized and its benefits are not thoroughly explored.

CONFLICT OF INTEREST

The author confirms that this article content has no conflicts of interest.

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ABBREVIATIONS

8-OHdG = 8-hydroxydeoxyguanine
ACPA = Antibodies against cyclic citrullinated peptide
DAS28 = Disease activity score in 28 joints
APC = Antigen presenting cells
CVD = Cardiovascular disease
Nox = Nicotinamide adenine dinucleotide phosphatoxidase
CAT = Catalase
GSH = Reduced glutathione
GPx = Glutathione peroxidase
GST = Glutathione S-transferase
HLA = Human leukocyte antigen
ROS = Reactive oxygen species
RA = Rheumatoid arthritis
SOD = Superoxide dismutase
TNFα = Tumor necrosis factor α
NF-κB = Nuclear factor kappa B
MtDNA = Mitochondrial DNA
RANK = Receptor activator of NF-κB
ICAM-1 = Intercellular adhesion molecules-1
VCAM-1 = Vascular cell adhesion molecule-1

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