Hydrogen promotes the activation of Cu, Zn superoxide dismutase in a rat corneal alkali-burn model

Takeshi Arima¹,², Tsutomu Igarashi¹, Masaaki Uchiyama¹, Maika Kobayashi¹, Ikuroh Ohsawa³, Akira Shimizu², Hiroshi Takahashi¹

¹Department of Ophthalmology, Nippon Medical School, Tokyo 113-8602, Japan
²Department of Analytic Human Pathology, Nippon Medical School, Tokyo 113-8602, Japan
³Biological Process of Aging, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan

Correspondence to: Takeshi Arima. Department of Ophthalmology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan. takesuiii0714@nms.ac.jp

Received: 2019-09-26 Accepted: 2020-05-12

Abstract

• AIM: To investigate the effects of hydrogen (H₂) on Cu, Zn superoxide dismutase (SOD1) activation in a rat model of corneal alkali burn.

• METHODS: In each rat, one cornea was subjected to alkali exposure. Physiological saline (saline group) or H₂-dissolved saline (H₂ group) was instilled continuously on the cornea for 5 min before and after alkali exposure. Inflammatory cells, neovascularization, and cytoplasmic SOD1 levels were evaluated immunohistochemically in enucleated eyes from both groups. Three-dimensional ultrastructural tissue changes in the eyes were analyzed using low-vacuum scanning electron microscopy.

• RESULTS: The numbers of both inflammatory and vascular endothelial cells were significantly reduced in the corneas of the H₂ group (P<0.01). Furthermore, H₂ treatment increased both cytoplasmic SOD1 levels (P<0.01) and activity in corneal epithelial cells (P<0.01). Notably, the SOD1 activity level in the H₂ group was approximately 2.5-fold greater than that in the saline group.

• CONCLUSION: H₂ treatment suppresses inflammation and neovascularization in the injured cornea and indirectly suppresses oxidative insult to the cornea by upregulating the SOD1 enzyme protein level and activity.

• KEYWORDS: hydrogen; alkali burn; Cu, Zn superoxide dismutase; low-vacuum scanning electron microscopy; rats

DOI:10.18240/ijo.2020.08.01

INTRODUCTION

Since we initially proposed a role for hydrogen (H₂) as a therapeutic antioxidant via the selective reduction of cytotoxic oxygen radicals in 2007¹, several studies have demonstrated the usefulness of H₂ and suggested its potential in therapeutic applications⁴. Accordingly, the field of H₂ medicine is rapidly growing, and more than 25 clinical studies (including double-blind clinical trials) are currently evaluating the therapeutic effectiveness of H₂¹⁴. More specifically, ophthalmology researchers have reported the applications of H₂ to directly reduce oxidative stress in the contexts of retinal artery occlusion⁵, corneal alkali burn⁶, and phacoemulsification cataract surgery⁷. Notably, however, H₂ has also been reported to suppress oxidative stress indirectly via other pathways⁸¹².

Activation of the cytoplasmic Cu, Zn superoxide dismutase (SOD1) enzyme, which is involved in antioxidant stress, is one pathway identified in studies of H₂ medicine¹³. SOD1 regulates reactive oxygen species levels, thus playing an important role in tissue homeostasis. Reports have demonstrated the involvement of H₂ in SOD1 activity and suggested that the former indirectly suppressed antioxidant stress²⁹. To date, however, few reports have described the effects of H₂ in the activation of SOD1 in an ophthalmological context.

Therefore, in the current study, we evaluated the effects of H₂ on inflammation and neovascularization, as well as the indirect effects on oxidative stress, by clarifying the influences of H₂ on SOD1 activity in the corneal alkali burn model.

MATERIALS AND METHODS

Ethical Approval All animal-based experiments were conducted in compliance with the Experimental Animal Ethics Review Committee of Nippon Medical School (No.29-055). All procedures conformed with the guidelines of the Association for Research in Vision and Ophthalmic and Visual Research.
Hydrogen activate SOD1 in rat corneal alkali-burn model

Animals  Eight-week-old male Wistar rats weighing 200 g were obtained from Sankyo Laboratory Service (Tokyo, Japan). Rats were housed in a specific pathogen free environment with a 12h light/12h dark cycle. Water and food were available and continuous clinical care (24hr per day/7d per week) was provided throughout the experiment to ensure prompt intervention when needed.

Methods  
Alkali burn model and preparation of H2-dissolved saline
One eye of each rat (n=104 in total) was subjected to an alkali burn under 3.5% isoflurane inhalation anesthesia, according to the following protocol: a circular piece of filter paper (diameter: 3.2 mm) soaked in 1 mol/L NaOH was placed on the central cornea for 1min. The corneas were rinsed with physiologic saline (saline group: n=39) or H2-dissolved saline (H2 group: n=39) for 5min prior to alkali exposure, and again after alkali exposure. In each rat, the uninjured normal cornea was used as a control.

H2-dissolved saline was prepared as described in our previous report[7]. Briefly, commercially available saline plastic bags (Otsuka Pharmaceutical, Tokyo, Japan) were placed in an acrylic vacuum chamber filled with H2 gas for 24hr (Figure 1A). Prior to use, the dissolved concentration of H2 in each bag was confirmed using a needle-type H2 sensor (Unisense, Aarhus, Denmark). In all experiments, H2-dissolved saline was used within 5min after retrieval from the vacuum chamber.

Rats were euthanized by exsanguination under 3.5% isoflurane inhalation anesthesia at 6h, 1, and 7d after alkali injury. The enucleated eyes were subjected to an alkali burn model and preparation of H2-dissolved saline

Histological and immunohistochemical analysis and LV-SEM observation
The enucleated eyes (n=8 for each group/for each endpoint) were fixed in 10% buffered formalin and embedded in paraffin prior to light microscopic observation. Subsequently, deparaffinized tissue sections (thickness: 2.5 μm) were stained with hematoxylin and eosin (HE), Naphthol AS-D chloroacetate esterase (EST) to detect infiltrating neutrophils[14], and LV-SEM. For the latter, tissues were stained with periodic acid-methenamine silver (PAM) to specify collagen[15-16].

For the immunohistochemical analysis of inflammatory cells, neovascularization, and SOD1 enzyme levels, the number of positive cells per sample was measured pathologically in high-power fields (magnification: 400×) located in two corneal areas (center: 3 fields, periphery: 2 fields). The following primary antibodies were used: 1) monoclonal mouse anti-aminopeptidase P antibody (JG12; Thermo Scientific, Rockford, IL, USA) to detect vascular endothelial cells[17], 2) monoclonal mouse anti-8-OHdG antibody (JaICA, Shizuoka, Japan) to detect DNA oxidative stress[18], 3) monoclonal mouse anti-CD68 antibody (ED1; BMA, Nagoya, Japan) to detect pan-macrophages, 4) monoclonal mouse anti-CD163 antibody (ED2; BMA, Nagoya, Japan) to detect M2 macrophages, and 5) polyclonal rabbit anti-SOD1 antibody (Stressgen, Victoria, BC, Canada) to detect SOD1 enzyme.

RESULTS
H2 Concentration in the Solution  After a 24-hour exposure to 100% H2 gas in an acrylic vacuum chamber (Figure 1A), the saline bag contained a dissolved H2 concentration of 87.2%.

In other words, the H2 gas easily penetrated the plastic bag, and the concentration was nearly equilibrated with the ambient H2 gas within 24h. After retrieval from the chamber, however, the H2 concentration in the bag decreased to 61.2% over 30min (Figure 1B). Initially, the H2 concentration at the exit of the infusion tube was 53.8% under continuous irrigation (10 mL/min) but decreased to 47.2% in 30min (Figure 1B).

Effects of H2 on Tissue Damage  HE staining revealed various inflammatory cells in the corneal limbus on day 1. Although these cells visibly infiltrated the center of the cornea on day 7 in the saline group, such changes were remarkably minimal in the H2 group (Figure 2A). Similarly, anterior segment photography showed severe hyphema and corneal opacity in the saline group on day 7, with minimal changes in the H2 group (Figure 2B).

Effects of H2 on Corneal Neovascularization  Vascular endothelial cell-specific JG12 staining was used to evaluate neovascularization (Figure 3A). Specifically, the degree of neovascularization was assessed by counting the labeled capillary lumens in a microscopic field. In the H2 group, neovascularization was suppressed by approximately 60% on day 7 relative to the saline group (Figure 3B).

Effects of H2 on Inflammatory Cells  Figure 4A depicts EST-positive neutrophils in the limbus at 6h and in the central cornea on day 1 and 8-OHdG-positive cells in the central cornea on day 1. Here, EST-positive neutrophils infiltrated the
area immediately proximal to the 8-OHdG-positive corneal stromal cells, suggesting the involvement of free radicals. At both time points, significantly smaller numbers of neutrophils were observed in the H₂ group, compared to the saline group (Figure 4B). Furthermore, LV-SEM revealed structural changes in the stromal collagen fibers, as well as a smaller number of inflammatory cells within these fibers in the H₂ group, compared to the saline group (Figure 4C).

Figure 1 Preparation of H₂-dissolved saline and measurement of H₂ concentration A: Saline bags were placed in an acrylic vacuum chamber in which the air was replaced with 100% H₂ gas; B: H₂ concentrations in the saline bags and at the exit of the infusion tube within 30 min. The full line indicates the H₂ concentration in the saline bags and the broken line indicates the H₂ concentration at the exit of the infusion tube.

Figure 2 Effects of H₂ instillation on rat corneal alkali injuries A: The histological effects of H₂ treatment were investigated in HE-stained corneal sections; B: A comparison of anterior segment photography of eyes from the H₂ and saline groups on day 7. n=20/104 in total, n=5 for each group/for each endpoint.

Figure 3 Evaluation of corneal neovascularization in response to H₂ treatment A: Immunohistochemical evaluation of JG12-positive vascular endothelial cells (arrow) in the corneal center on day 7; B: The capillary lumens in the corneal center were significantly reduced by H₂ treatment. n=48/104 in total, n=8 for each group/for each endpoint. bP<0.01.
Additionally, ED1 and ED2 immunostaining were used to detect the infiltration of pan-macrophages and M2 macrophages, respectively (Figure 5A). Notably, H2 significantly reduced the infiltration of ED1-positive pan-macrophages while significantly increasing that of ED2-positive M2 macrophages (Figure 5B and 5C).

**Effects of H2 on SOD1 enzyme activity** To further explore the potential effects of H2 on anti-oxidant enzymes, we investigated the protein levels of SOD1. Notably, H2 significantly reduced the infiltration of ED1-positive pan-macrophages while significantly increasing that of ED2-positive M2 macrophages (Figure 5B and 5C).

**DISCUSSION**

Strong inflammation and subsequent neovascularization are often observed in the alkali-burned cornea [21-22]. Accordingly, the observed effects of H2 have led to considerable interest in its application to alkali-induced corneal injury. Treatment with H2 contributes to the protective effect against inflammatory injury by selectively reducing hydroxyl radical, the most cytotoxic of reactive oxygen species [23-24]. Previously, Kubota et al [6] evaluated the effect of H2 in a mouse model involving an alkali burn in the SOD1-deficient cornea. Their findings proved the oxidative nature of the alkali burn injury and demonstrated...
the anti-angiogenic effects of H2 in the cornea. In our current study, our observation of the anti-angiogenic effect of H2 agrees with the reported findings by Kubota et al[6]. In our experiments, immunohistochemical staining for JG12 revealed the significant suppression of neovascularization in the H2 group. We additionally conducted a detailed analysis of the inflammatory cells infiltrating the injured corneal tissues. Immunohistochemical staining revealed a significantly smaller number of infiltrating neutrophils in the H2 group relative to the saline group at all time points after injury. We further observed an association between infiltrating neutrophils and 8-OHdG-positive cells and observed that H2 significantly suppressed both populations. As H2 acts as a free radical scavenger, our findings indicate that a reduction in oxidative stress might help to suppress the inflammatory cell invasion, consistent with a previous report[6]. We further confirmed this effect via observing corneal collagen using LV-SEM, a modality widely used to evaluate three-dimensional ultrastructural changes in tissues specimens intended for light microscopy[19,25-26]. H2 helped to maintain the normal alignment of stromal collagen fibers, an important factor in corneal transparency[27].

We also observed a significant effect of H2 on macrophage infiltration. Specifically, treatment with H2 significantly reduced the infiltration of ED1-positive pan-macrophages while significantly increasing the infiltration of ED2-positive M2 macrophages. Notably, M1 macrophages are inflammatory cells, whereas M2 macrophages resolve inflammation and thus play critical roles in tissue remodeling and wound healing. Previous reports of corneal studies indicate that M2 macrophages function in the wound repair process in this tissue[21,28]. Therefore, our observation that H2 treatment promotes M2 macrophage expression suggests another potential clinical application of this therapy. However, further experiments are needed to elucidate the pathological mechanism underlying the M1/M2 balance.

SODs are well-known players in antioxidant stress pathways. Of these enzymes, SOD1 is expressed in most tissues and is responsible for 90% of SOD activity[13]. In SOD1-deficient animals, free radical-induced injuries lead to degenerative or inflammatory diseases[29-30], and the observation of enhanced corneal alkali burn injury suggested the importance of SOD1 as an anti-free radical effector[6]. In contrast, the use of instillation to abundantly increase the level of exogenous SOD1 in the eye appeared to reduce inflammation by resolving oxidative stress[31]. Murakami et al[32] have reported that treatment with H2 indirectly reduces oxidative stress by inducing SOD through Nrf2 pathway. In the present study, therefore, we hypothesized that SOD1 might be involved in mechanism by which H2 indirectly suppresses free radicals. Consistent with this hypothesis, we observed cytoplasmic SOD1 activation in response to H2 in corneal epithelial cells even in the early stages of wound healing. Taken together, these results suggest that the anti-oxidative effects of H2 involve not only a direct free radical scavenging effect, but also the activation of SOD1, consistent with a previous neurological study wherein H2 was recognized as an effective treatment candidate for amyotrophic lateral sclerosis involving a mutation of cytosolic SOD1[33].

In summary, our study of alkali-induced corneal injury revealed that H2 exerts its anti-inflammatory effects not only by suppressing free radicals, but also by inducing the activation of SOD1. Further understanding of these characteristic effects of H2 may enable additional ophthalmologic treatment strategies.

Figure 6 Increased cytoplasmic SOD1 expression in response to H2 treatment. A: Representative images of SOD1 (arrow)-immunostained corneal epithelial cells during wound healing after alkali injury; B: Bar chart demonstrating the activation of SOD1 in response to H2 administration over time; C: Bar chart demonstrating that cytoplasmic SOD1 expression in the central cornea was significantly upregulated in the H2 group vs the saline group and normal corneas at 6h post-injury. n=30/104 in total, n=5 for each group/for each endpoint. bP<0.01.
Hydrogen activate SOD1 in rat corneal alkali-burn model

ACKNOWLEDGEMENTS

Conflicts of Interest: Arima T, None; Igarashi T, None; Uchiyama M, None; Kobayashi M, None; Ohsawa I, None; Shimizu A, None; Takahashi H, None.

REFERENCES


