# Hydrogen and N-acetyl-L-cysteine rescue oxidative stress-induced angiogenesis in a mouse corneal alkali-burn model

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Word count: 2949 words.

Corresponding author: Shigeto Shimmura, MD. Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan Phone: +81-3-5363-3269, FAX: +81-3-3358-5961 E-mail: shige@sc.itc.keio.ac.jp **Purpose:** To investigate the role of reactive oxygen species (ROS) as the prime initiators of the angiogenic response following alkali injury of the cornea, and observe the effects of anti-oxidants in preventing angiogenesis.

**Methods:** The corneal epitheliums of SOD-1 deficient mice or wild type mice (WT) were removed after applying 0.15N NaOH to establish the animal model of alkali burn. ROS production was semi-quantitatively measured by dihydroethidium (DHE) fluorescence. Angiogenesis was visualized by CD31 immunohistochemistry. The effects of the specific NF- $\kappa$ B inhibitor DHMEQ, the antioxidant N-acetyl-L-cysteine (NAC) and hydrogen (H<sub>2</sub>) solution were observed.

**Results:** ROS production in the cornea was enhanced immediately after alkali injury as shown by increased dihydroethidium (DHE) fluorescence (p < 0.01). NFkappa B (NF- $\kappa$ B) activation and the upregulation of vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) were significantly enhanced (p < 0.01), leading to a significantly larger area of angiogenesis. Angiogenesis in SOD-1<sup>-/-</sup> mice corneas were significantly higher in wild type mice (P < 0.01), confirming the role of ROS. Pretreatment with the specific NF- $\kappa$ B inhibitor DHMEQ or the antioxidant N-acetyl-L-cysteine (NAC) significantly reduced corneal angiogenesis by down regulating the NF- $\kappa$ B pathway (p < 0.01) in both WT and SOD-1<sup>-/-</sup> mice. Furthermore, we showed that irrigation of the cornea with hydrogen (H<sub>2</sub>) solution significantly reduced angiogenesis after alkali-burn injury (p < 0.01).

**Conclusion**: Immediate anti-oxidant therapy with H<sub>2</sub>-enriched irrigation solution is a new potent treatment of angiogenesis in cornea to prevent blindness due to alkali burn.

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#### IOVS

Although the cornea is a physiologically transparent tissue, pathological corneal neovascularization (NV) is observed following inflammation due to infection, aberrant immune responses or chemical/ thermal burns. The balance between angiogenic and anti-angiogenic factors determine the fate of corneal NV during wound healing. <sup>1-4</sup> It is widely known that alkali burns cause acute inflammation and NV in the cornea, and alkali burns in animals are often used as a NV model. <sup>5, 6</sup> However, the mechanisms involved in the induction of corneal NV is complex, with a long list of angiogenic growth factors such as fibroblast growth factor (TGF), vascular endothelial growth factor (VEGF) and transforming growth factor (TGF), in addition to chemokines that are mobilized following injury to the cornea <sup>1</sup>. Therefore, present therapeutic strategies for corneal NV have focused on inhibiting VEGF, the key trigger of pathological corneal NV, <sup>7</sup> or blocking its receptors. <sup>6</sup> However, from a therapeutic standpoint, initial therapy to prevent the onset of corneal NV is far more effective and efficient. We therefore hypothesized that oxidative stress due to reactive oxygen species (ROS) at the onset of injury can be an upstream target for therapeutic intervention.

ROS include highly reactive molecules such as the superoxide radical ( $O_2^{-}$ ), hydrogen peroxide ( $H_2O_2$ ), and the hydroxyl radical ( $\cdot$ OH). ROS play an important role in cellular homeostasis, while pathologically high concentrations are involved with cell death, disease and aging. ROS can activate the transcription factor NF- $\kappa$ B, <sup>8</sup> which then translocates to the nucleus to induce the expression of inflammatory cytokines such as VEGF, MCP-1, ILs, and TNF $\alpha$  <sup>9</sup>. These cytokines not only induce corneal NV, but can also recruit inflammatory cells that further exacerbate inflammation causing further tissue damage. In this study, we demonstrate how ROS can directly trigger pathological corneal NV in an animal model, and more importantly, show that the topical use of H<sub>2</sub>

water has a prophylactic effect against pathological blood vessels invading into the clear cornea.

## MATERIAL AND METHODS

## Alkali burn model

Male ICR mice at the age of 6 to 7 weeks were purchased from CLEA Japan, Inc (Japan) and SOD-1 deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the study was approved by the institutional board on the use of animals. Corneal NV was induced by alkali injury. Briefly, after general anesthesia with pentobarbital (Nembutal, 50 mg/kg), 2  $\mu$ L of 0.15 M NaOH was applied onto the corneal surface. Subsequently, total corneal limbus and epithelium were scraped off with a surgical blade under a microscope. Ofloxacin ophthalmic ointment was instilled immediately after the operation.

## Treatment with NAC or DHMEQ

From 3 days before the alkali burn, wild type and SOD-1<sup>-/-</sup> mice were treated with an N-Acetyl 1-L-cysteine (NAC; Nakarai tesque, Japan), or a specific NF- $\kappa$ B inhibitor (DHMEQ; dehydroxymethylepoxyquinomicin) or vehicle (0.083% dimethyl sulfoxide [DMSO] in phosphate buffered saline [PBS]) daily for 6 days. NAC and DHMEQ were injected into mice intraperitoneally. DHMEQ is a novel NF- $\kappa$ B inhibitor, based on the structure of epoxyquinomicin C, which was originally isolated from Amycolatopsis. DHMEQ has been shown to inhibit nuclear translocation of NF- $\kappa$ B without affecting

phosphorylation and degradation of I- $\kappa$ B $\alpha$ . Mice received NAC at a dose of 200 mg/kg or DHMEQ at 5 mg/ kg body weight.

## Treatment with H<sub>2</sub> water

In order to observe the prophylactic effects of  $H_2$  water, a potent anti-oxidant found effective in the prevention of ischemic brain injury <sup>10</sup>, eyes following alkali burns were irrigated with  $H_2$  water (Blue Mercury Inc, Japan) for 30 min.  $H_2$  water was rendered isotonic by diluting 20x concentrated PBS 1:20 prior to irrigation, where the final  $H_2$ concentration was in the range of 0.5 to 0.6 ppm. PBS alone was used as vehicle treatment, and untreated corneas were used as control.

## Quantification of corneal angiogenesis

Six days after alkali burn, mice were sacrificed. Enucleated eyes were further fixed with ice-cold 0.5% PFA for 10 min, followed by ice-cold methanol treatment for 70%-20 min, 80%-20 min, 100%-20 min. After washing steps in PBS and blocking with TNB buffer {1N Tris-HCL (pH 7.5) 10 ml, DW 90 ml, NaCl 0.87 g, Blocking reagent 0.5 g (FP1020, Perkin Elmer Life Science, Inc., MA), 20% triton 0.5 ml} in 0.1% triton for 2hrs, the whole corneas were stained over night at 4 °C with purified rat anti-mouse CD31 (PECAM-1) (1:300, BD; 550274), and washed and further incubated with Alexa flour 488 goat anti-rat IgG (1:300, Life technologies, CA) for 1 hour at RT. The flat mounts were imaged with a fluorescence microscope (BIOREVO BZ-9000, KEYENCE, Japan), and quantified by ImageJ program (National Institute of Health, Bethesda, MD). The area of corneal neovascularization was calculated by the following equation where the "total area" is the area within the limbal vessel arcade and the "avascular area" is the

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area of remaining clear cornea.

Area of NV (%) = (total area - avascular area)/total area x 100

### **Measurement of ROS**

Eyes were enucleated and immediately frozen in OCT compound (Sakura Finetek, Torrance, CA) as soon as 0.15 N NaOH dropped and washed out. Unfixed cryosections (10  $\mu$ m) were incubated with 5  $\mu$ M dihydroethidium (DHE; Molecular Probes, Eugene, OR) for 15 minutes at 37°C at room temperature, as previously reported. <sup>11, 12</sup> Sections were examined using a microscope equipped with a digital camera (Carl Zeiss), and the intensity of the staining was measured using the ImageJ program. In order to facilitate the detection of ROS by image analysis, the epithelium was left intact for this assay.

## Immunohistochemical staining

Eyes were enucleated and fixed in 4% PFA overnight at 4 °C. After fixation, tissues were processed and embedded in an OCT compound, frozen in liquid nitrogen, and stored at -80°C until sectioning. Frozen 6 µm to 8 µm-thick sections were cut with a cryostat and mounted on slides. We used the anti-mouse CD31 antibody (rat monoclonal, clone MEC 13.3, 550274, BD Pharmigen) for detecting blood vessels in the cornea, or anti- mouse F4/80 antibody (rat monoclonal, clone CI: A3-1, MCA497R, AbD serotec, Raleigh, NC) for macrophages. Alexa Fluor 488 or 546 -conjugated goat anti-rat antibodies were used as secondary antibody. Nuclei were counterstained with Hoechst 33342. The increase in number of F4/80 positive cells per field was calculated at day 0, 3 and 6 after injury.

## Enzyme-linked immunosorbent assay

Three days after alkali burn, mice were sacrificed with an overdose of anesthesia, and the eyes were immediately enucleated. The whole cornea including limbus, epithelium, stroma and endothelium was isolated and placed into 100uL lysis buffer (0.02 M HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid], 10% glycerol, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 100 μM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton, 100 mM NaF, 4 mM EDTA [ethylenediaminetetraacetic acid], pH 8.0) supplemented with protease inhibitors (2 mg/L aprotinin, 100  $\mu$ M phenylmethylsulfonyl fluoride, 10  $\mu$ M leupeptin, 2.5  $\mu$ M pepstatin A) and sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4 °C. VEGF and MCP-1 levels in the supernatant were determined with the mouse VEGF and MCP-1 ELISA kits (R&D Systems, Minneapolis, MN), respectively, according to the manufacturer's protocols. Similarly, phosphorylated NF- $\kappa$ B p65 levels were measured with the phosphorylated NF- $\kappa$ B p65 ELISA kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA).

## **Quantitative RT-PCR**

Animals were sacrificed with an overdose of anesthesia. The eyes were immediately enucleated and the cornea (including epithelium, stroma, endothelium and limbus) was carefully isolated. Total RNA was extracted from the cornea using an extraction reagent (TRIzol; Life Technologies), and cDNA was synthesized with First-Strand c-DNA Synthesis Kit (GE Healthcare). For the RT-PCR reaction, mRNA transcripts were detected using the TaqMan real-time quantitative RT-PCR procedure (TaqMan Fast Universal PCR Master Mix Reagents Kit; Applied Biosystems). The RT-PCR assay was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems). The threshold cycle (Ct) was calculated by the instrument's software (7500 Fast System). A quantitative PCR assay for mRNA of f4/80 and mcp-1 were performed using TaqMan Gene Expression Assay Mix (Mm00802530\_m1 and Mm00441242\_m1, respectively).

## **Statistical analyses**

All results were expressed as mean  $\pm$  standard deviation (SD). The values were processed for statistical analyses (Mann-Whitney test) or Unpaired Student's t-test and differences were considered statistically significant at p < 0.05.

## RESULTS

## Alkali burn enhances oxidative stress in the cornea

To elucidate the contribution of ROS in alkali-burned injury, we examined ROS induction by administration of NaOH using dihydroethidium (DHE) assay, where DHE reacts with  $O_2^-$  to become fluorescent. Application of 0.15N NaOH to the cornea induced significant increase of ROS in the epithelium (2.31 ± 0.51-fold of untreated control; p < 0.01) (Fig. 1A, B). Alkali burn induced angiogenesis (PECAM-1) and recruited macrophages (F4/80), which were not observed in untreated controls (Fig. 1C). F4/80 positive macrophages significantly increased by day 3 and day 6 (p < 0.05).

# Alkali burn-induced corneal NV was inhibited by the antioxidant NAC or the NF-κB inhibitor DHMEQ.

Increased oxidative stress in the alkali-burned cornea suggested that oxidative stress induced by alkali injury was the main trigger for corneal angiogenesis. Therefore, we examined corneal NV in mice treated with the antioxidant, NAC. As expected, antioxidant treatment significantly suppressed alkali-burn induced corneal NV (p < 0.01). Furthermore, inhibition of NF- $\kappa$ B, a transcription factor downstream of oxidative stress, suppressed corneal NV as well (p < 0.01) (Fig. 2A, B).

To investigate the molecular mechanisms involved, we examined NF- $\kappa$ B phosphorylation and VEGF expression in alkali-burned corneas. A statistically significant increase in NF- $\kappa$ B phosphorylation (p < 0.01) (Fig. 2C), and VEGF expression (p < 0.01) was induced by alkali injury, which was inhibited by the administration of NAC or DHMEQ (Fig. 2D). Corneal NV induced by various kinds of stimuli, including alkali burn, is accompanied with and accelerated by infiltrating macrophages <sup>13</sup>. To quantify macrophage infiltration to the cornea, we measured mRNA levels of the macrophage marker f4/80. We found that f4/80 mRNA in alkali-burned corneas six days after injury decreased with NAC (p < 0.05) or DHMEQ (p < 0.05) treatment (Fig. 2E). Similarly, alkali burn-induced increase of mcp-1, a chemotactant for macrophage infiltration <sup>14</sup>, was also suppressed by NAC (p < 0.05) or DHMEQ (p < 0.05) (Fig. 2F). These results indicated antioxidant treatment or inhibition of NF- $\kappa$ B suppressed alkali-burned corneal NV via down regulation of VEGF as well suppression of macrophage infiltration.

# SOD-1<sup>-/-</sup> mice show enhanced corneal NV

In order to confirm the role of ROS in corneal angiogenesis induced by alkali burn, we compared NV areas in SOD-1<sup>-/-</sup> mice compared with littermate wild type controls. The NV area in SOD-1<sup>-/-</sup> mice (43.63 ± 13.42%, n = 6) was significantly larger compared with SOD-1<sup>+/+</sup> control (30.11 ± 7.16%, n=6, p < 0.01) (Fig. 3A, B). This shows that loss of SOD-1 function enhanced alkali burn-induced corneal NV. The expression of *f*4/80 was significantly increased in SOD-1<sup>-/-</sup> compared with SOD-1<sup>+/+</sup> (p < 0.01) (Fig. 3C). Furthermore, VEGF protein was significantly increased in SOD-1<sup>-/-</sup> compared in SOD-1<sup>-/-</sup> compared with SOD-1<sup>+/+</sup> (p < 0.01) (Fig. 3D). These results suggest that the anti-oxidant effect of SOD-1 suppresses corneal angiogenesis by inhibiting macrophage infiltration and VEGF up regulation following alkali-burns.

# NAC and DHMEQ rescues increased corneal NV in SOD-1<sup>-/-</sup> mice

Since the loss of SOD-1 expression caused an increase in macrophage infiltration, VEGF expression and neovascularization, we examined if NAC can rescue the phenotype and whether NF- $\kappa$ B was involved. As expected, we found that both NAC (Fig. 4A, B) (p < 0.05) and DHMEQ (Fig. 4C, D) significantly decreased NV area (p < 0.01) indicating that the NF- $\kappa$ B pathway was activated by the increase in ROS associated with the loss of SOD-1 protein.

## Post-trauma irrigation with H<sub>2</sub> water suppresses corneal NV

We found that pre-treatment with NAC can prevent NV formation in the cornea. Since pretreatment is not possible in a clinical setting, we further sought to find a rescue protocol that is effective after alkali injury. The most important emergency procedure following corneal chemical burns is meticulous irrigation of the ocular surface using saline solutions to dilute residual chemicals in the tissue. We therefore hypothesized that irrigating with H<sub>2</sub> water, a recently recognized antioxidant, instead of saline will enhance the prophylactic effects against ensuing inflammation and corneal NV. NAC was not used in the irrigation experiment due to its acidic pH. As shown in Fig. 5, irrigating with isotonic H<sub>2</sub> water significantly reduced ROS associated DHE fluorescence (p < 0.05) (Fig. 5A, B) and NF- $\kappa$ B phosphorylation (p < 0.05) (Fig. 5C). More importantly, eyes irrigated with H<sub>2</sub> water also had significantly smaller NV areas (p < 0.01) (Fig. 6A, B) that were associated with lower VEGF (p < 0.01) (Fig. 6C) and MCP-1 (p < 0.01) (Fig. 6D) protein levels compared to PBS alone.

## DISCUSSION

In the present study, we showed that oxidative stress triggered angiogenesis through activation of the NF- $\kappa$ B pathway using a corneal alkali burn model. The role of ROS was confirmed using SOD-1<sup>-/-</sup> mice, which showed increased VEGF protein levels and accumulation of macrophages that further accelerated corneal NV (Fig. 3). While angiogenesis induced by alkali burn is not recognized as an oxidative stress model, we also examined corneal NV by ultraviolet B radiation (UVB), a more common source of oxidative stress. SOD-1<sup>-/-</sup> mice had significantly more corneal NV compared to wild type control following exposure to UVB (supplementary figure). The current understanding of the alkali burn model of angiogenesis is that inflammation is the trigger of angiogenesis following injury. Therefore, most studies have focused on anti-inflammatory agents such as NF- $\kappa$ B inhibitors <sup>5, 13</sup>, anti-VEGF agents <sup>15, 16</sup> and inhibition of the VEGF receptor <sup>6</sup>. Corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDS) are also used clinically to suppress inflammation, however, these

agents are non-specific with side effects such as increased ocular pressure. We also examined the effects of the specific NF- $\kappa$ B inhibitor, DHMEQ, and confirmed that inhibiting NF- $\kappa$ B reduces VEGF protein levels, *mcp-1* expression, as well as NV area following alkali burns (Fig. 2). Nuclear translocation of NF- $\kappa$ B up regulates *mcp-1* and *vegf* leading to increased macrophage infiltration and angiogenesis, respectively. <sup>9</sup> Compared to other NF- $\kappa$ B inhibitors reported in the literature, DHMEQ is unique in that it blocks the translocation of NF- $\kappa$ B p65 into the nucleus <sup>17</sup>, and is therefore highly specific to this pathway.

However, the most important finding of our study is the fact that direct ROS formation by alkali injury precedes the inflammatory response. ROS-related DHE fluorescence was enhanced immediately following alkali burn (Fig. 1A). Since oxidative stress was upstream of the cascade leading to corneal NV, we hypothesized that supplementing exogenous antioxidants such as NAC may protect cornea from alkali burn-induced angiogenesis. As expected, pretreatment with NAC significantly reduced the angiogenic response following alkali burns as shown in Fig. 2. NAC inhibited VEGF production, *f4/80* and *mcp-1* expression, as well as NV comparable to the effects by DHMEQ, demonstrating the potency of ROS in the activation of NF- $\kappa$ B. Of particular note is the fact that NAC treatment was only done up to 2 days following injury, indicating that early intervention with antioxidants was particularly important. Recent studies have also shown that oxidative stress promotes pathological events via NF- $\kappa$ B activation<sup>18, 19</sup>, and that NF- $\kappa$ B is indeed a redox-sensitive transcriptional factor<sup>8</sup>.

Since pre-treatment with antioxidants in not feasible clinically, we further investigated the possibility of using  $H_2$  water as an antioxidant irrigation solution

following injury. H<sub>2</sub> water irrigation significantly quenched ROS-related DHE fluorescence and NF- $\kappa$ B phosphorylation (Fig. 5). Furthermore, the 30-minute irrigation period was sufficient to significantly reduce VEGF and MCP-1 protein levels, as well as the final area of corneal NV. H<sub>2</sub> gas is a harmless gas by itself, and is used as a diluent gas for deep sea divers to improve ventilatory mechanics and to attenuate the excitatory effects of high pressure on the central nervous system <sup>20</sup>. Recently, Ohsawa et, al. introduced H<sub>2</sub> in the field of medicine as a specific inhibitor of the hydroxyl radical, a particularly reactive ROS species <sup>10</sup>. Hydrogen reduces the hydroxyl radical to H2O2, thereby preventing subsequent lipid peroxidation, DNA oxidation and mitochondrial dysfunction. They reported that inhalation of H<sub>2</sub> in a rat model of cerebral ischemia and reperfusion model significantly reduced ROS-induced brain injury. Similar reports since then showed similar protective effects H<sub>2</sub> against ROS damage in a neonatal rat model, <sup>21</sup> an intestinal graft injury model, <sup>22</sup> and drug-induced nephrotoxicity model. <sup>23</sup> Unlike other antioxidants, H<sub>2</sub> can diffuse directly through tissue due to its small molecular size, and does not require specific carriers or membrane channels <sup>10</sup>.

Chemical burn injuries of the cornea are caused by various toxic chemicals used mostly in the industry. Alkali injuries are the most severe, causing blindness in the working age population, with an incidence ranging from 7.0 to 9.9% of all ocular traumas. <sup>24-26</sup> Alkaline was the most common causative agent (66.7%) among chemical injuries in a series from the UK. <sup>27</sup>The incidence is probably higher in developing countries based on the number of surgical procedures performed. <sup>28</sup> Prompt irrigation of the injured eye at the site of injury as well as in the emergency clinic is vital in order to prevent blindness, since advanced stages are more difficult to treat due to stem cell deficiency and corneal angiogenesis. In conclusion, our report shows that oxidative stress is a direct result of alkali injury, and that immediate action to quench reactive oxygen species can inhibit pathological angiogenesis. Furthermore, compared to using expensive reagents such as antibodies or anti-metabolites, H<sub>2</sub> water is inexpensive and safe to use in any clinical setting. We hope that our results will shed light on the importance of anti-oxidant therapy in alkali burns.

# ACKNOWLEDGMENTS

This study was partly supported by a Grant-in-Aid for Scientific Research (C) by the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

### REFERENCES

1. Chang JH, Gabison EE, Kato T, Azar DT. Corneal neovascularization. *Curr Opin Ophthalmol* 2001;12:242-249.

2. Huang AJ, Li DQ, Li CH, Shang TY, Hernandez E. Modulation of corneal vascularization. *Ocul Surf* 2005;3:S190-193.

3. BenEzra D, Griffin BW, Maftzir G, Sharif NA, Clark AF. Topical formulations of novel angiostatic steroids inhibit rabbit corneal neovascularization. *Invest Ophthalmol Vis Sci* 1997;38:1954-1962.

4. Lee P, Wang CC, Adamis AP. Ocular neovascularization: an epidemiologic review. *Surv Ophthalmol* 1998;43:245-269.

5. Saika S, Miyamoto T, Yamanaka O, et al. Therapeutic effect of topical administration of SN50, an inhibitor of nuclear factor-kappaB, in treatment of corneal alkali burns in mice. *Am J Pathol* 2005;166:1393-1403.

6. Mochimaru H, Usui T, Yaguchi T, et al. Suppression of alkali burn-induced corneal neovascularization by dendritic cell vaccination targeting VEGF receptor 2. *Invest Ophthalmol Vis Sci* 2008;49:2172-2177.

7. Bock F, Onderka J, Dietrich T, et al. Bevacizumab as a potent inhibitor of inflammatory corneal angiogenesis and lymphangiogenesis. *Invest Ophthalmol Vis Sci* 2007;48:2545-2552.

8. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 2000;408:239-247.

9. Baldwin AS, Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 1996;14:649-683.

10. Ohsawa I, Ishikawa M, Takahashi K, et al. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med* 2007;13:688-694.

11. Berent-Spillson A, Russell JW. Metabotropic glutamate receptor 3 protects neurons from glucose-induced oxidative injury by increasing intracellular glutathione concentration. *J Neurochem* 2007;101:342-354.

12. Sasaki M, Ozawa Y, Kurihara T, et al. Neuroprotective effect of an antioxidant, lutein, during retinal inflammation. *Invest Ophthalmol Vis Sci* 2009;50:1433-1439.

13. Miura Nakao S. Hata Y. M, et al. Dexamethasone inhibits interleukin-1beta-induced corneal neovascularization: role of nuclear factor-kappaB-activated stromal cells in inflammatory angiogenesis. Am J Pathol 2007;171:1058-1065.

14. Watanabe T, Higuchi K, Hamaguchi M, et al. Monocyte chemotactic protein-1 regulates leukocyte recruitment during gastric ulcer recurrence induced by tumor necrosis factor-alpha. *Am J Physiol Gastrointest Liver Physiol* 2004;287:G919-928.

15. Papathanassiou M, Theodossiadis PG, Liarakos VS, Rouvas A, Giamarellos-Bourboulis EJ, Vergados IA. Inhibition of corneal neovascularization by subconjunctival bevacizumab in an animal model. *Am J Ophthalmol* 2008;145:424-431.

16. Yoeruek E, Ziemssen F, Henke-Fahle S, et al. Safety, penetration and efficacy of topically applied bevacizumab: evaluation of eyedrops in corneal neovascularization after chemical burn. *Acta Ophthalmol* 2008;86:322-328.

17. Watanabe M, Dewan MZ, Okamura T, et al. A novel NF-kappaB inhibitor DHMEQ selectively targets constitutive NF-kappaB activity and induces apoptosis of multiple myeloma cells in vitro and in vivo. *Int J Cancer* 2005;114:32-38.

18. Pueyo ME, Gonzalez W, Nicoletti A, Savoie F, Arnal JF, Michel JB. Angiotensin II stimulates endothelial vascular cell adhesion molecule-1 via nuclear factor-kappaB activation induced by intracellular oxidative stress. *Arterioscler Thromb Vasc Biol* 2000;20:645-651.

19. Morigi M, Macconi D, Zoja C, et al. Protein overload-induced NF-kappaB activation in proximal tubular cells requires H(2)O(2) through a PKC-dependent pathway. *J Am Soc Nephrol* 2002;13:1179-1189.

20. Brauer RW, Way RO. Relative narcotic potencies of hydrogen, helium, nitrogen, and their mixtures. *J Appl Physiol* 1970;29:23-31.

21. Cai J, Kang Z, Liu WW, et al. Hydrogen therapy reduces apoptosis in neonatal hypoxia-ischemia rat model. *Neurosci Lett* 2008;441:167-172.

22. Buchholz BM, Kaczorowski DJ, Sugimoto R, et al. Hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury. *Am J Transplant* 2008;8:2015-2024.

23. Nakashima-Kamimura N, Mori T, Ohsawa I, Asoh S, Ohta S. Molecular hydrogen alleviates nephrotoxicity induced by an anti-cancer drug cisplatin without compromising anti-tumor activity in mice. *Cancer Chemother Pharmacol* 2009;64:753-761.

24. Jones NP, Hayward JM, Khaw PT, Claoue CM, Elkington AR. Function of an ophthalmic "accident and emergency" department: results of a six month survey. *Br Med J (Clin Res Ed)* 1986;292:188-190.

25. Pfister RR. Chemical injuries of the eye. *Ophthalmology* 1983;90:1246-1253.

26. Vernon SA. Analysis of all new cases seen in a busy regional centre ophthalmic casualty department during 24-week period. *J R Soc Med* 1983;76:279-282.

27. Macdonald EC, Cauchi PA, Azuara-Blanco A, Foot B. Surveillance of severe chemical corneal injuries in the UK. *Br J Ophthalmol* 2009;93:1177-1180.

28. Yao YF, Zhang B, Zhou P, Jiang JK. Autologous limbal grafting combined with deep lamellar keratoplasty in unilateral eye with severe chemical or thermal burn at late stage. *Ophthalmology* 2002;109:2011-2017.

## **FIGURE LEGENDS**

**Figure 1.** Alkali burns induce reactive oxygen species formation. (A) Sodium hydroxide (0.15 N NaOH) induced oxidative stress in mice cornea as shown by increased DHE fluorescence compared to controls. Scale bar = 100  $\mu$ m. (B) DHE fluorescence levels in the cornea were significantly higher in alkali-burned mice (2.31 ± 0.51-fold of control; \*\* *p* < 0.01, n=4). (C) Alkali burn induced immunoreactivity against PECAM-1 and F4/80, indicating angiogenesis and macrophage invasion. Arrow indicates normal vessels of the peripheral cornea. (D) F4/80 positive macrophages were significantly higher at day 3 (13.7 ± 7.4 cells/ field; *p* < 0.05, n=6) and day 6 (38.6 ± 11.4 cells/ field; *p* < 0.05, n=5) compared to day 0 (0.8 ± 0.8 cells/ field). Error bars indicate SD. Scale bar = 100 µm.

**Figure 2.** Corneal NV due to alkali burns is suppressed by NAC or DHMEQ. (A, B) Alkali burn induced corneal NV in mice cornea. NV area was significantly (\*\*p < 0.01; each) lower in NAC or DHMEQ treated mice (18.31 ± 4.25, 17.06 ± 5.99, n=8) than in vehicle-treated controls (35.98 ± 6.35, n=6) (day6). Scale bar = 500 µm. (C) NF- $\kappa$ B activation was significantly higher in alkali-burned mice, which was significantly suppressed by treatment with NAC or DHMEQ. n = 8-12 (day3). \*\* p < 0.01. (D) Similarly, VEGF protein levels were significantly elevated in alkali-burned mice, which were significantly suppressed by treatment with NAC or DHMEQ. n = 8-11 (\*\*p < 0.01; each) (day3). *f4/80* (**E**) and *mcp-1* (**F**) mRNA in alkali-burned mice was significantly elevated than controls, which was significantly suppressed by treatment with NAC or DHMEQ. n = 7-8 and 8-9 for *f4/80* (\*p < 0.05 each) and *mcp-1* (\*p < 0.05 each), respectively. Error bars indicate SD. **Figure 3.** Corneal NV is enhanced in SOD-1 (-/-) mice. (A, B) Corneal NV area was significantly (\*\*p < 0.01) higher in SOD-1<sup>-/-</sup> mice (43.63 ± 13.42) than in SOD-1<sup>+/+</sup> mice (30.11 ± 7.16) (day6), n=19 each. Scale bar = 500 µm. (C) *f4/80* mRNA levels in SOD-1<sup>-/-</sup> mice were significantly elevated than those in SOD-1<sup>+/+</sup> mice. *n* = 4 each. (Day6) \*\* *p* < 0.01. (D) VEGF protein levels in SOD-1<sup>-/-</sup> mice were significantly higher than those in SOD-1<sup>+/+</sup> mice. *n* = 5-6. \* *p* < 0.05 (day3). Error bars indicate SD.

**Figure 4.** Corneal NV in SOD-1<sup>-/-</sup> mice is suppressed by NAC and DHMEQ. (A, B) Corneal NV area in SOD-1<sup>-/-</sup> mice was significantly lower than control ( $45.85 \pm 8.67$ , n=11) when pretreated with NAC ( $38.53 \pm 7.19$ , n=13) starting 3 days prior to injury \* *p* < 0.05. (C, D) A similar reduction in NV area was observed in DHMEQ treated mice ( $26.78 \pm 7.81$ , n=13) compared to vehicle-treated controls ( $43.626 \pm 13.427$  n=19) \*\* *p* < 0.01 (day6). Error bars indicate SD. Scale bar = 500 µm.

**Figure 5** Corneal ROS formation is suppressed with H<sub>2</sub>. To examine oxidative stress as a potential stimulus for angiogenic and molecular inflammatory events, corneal ROS expression was analyzed by DHE fluorescence. (A, B) ROS-induced DHE fluorescence in the corneal epithelium was significantly higher in vehicle-treated alkali-burned mice  $(1.6 \pm 0.2 \text{ ratio of control}; * p < 0.05)$  than in untreated controls. DHE intensity was significantly suppressed in H<sub>2</sub> treated mice  $(0.6 \pm 0.3 \text{ ratio of control}; * p < 0.05)$  than in vehicle-treated alkali-burned mice  $(1.6 \pm 0.2 \text{ ratio of control})$  n = 4-5. Scale bar = 100 µm. (C) Phosphorylated NF- $\kappa$ B p65 levels were significantly (\*p < 0.05) higher in vehicle-treated alkali-burned mice  $(311 \pm 381 \% \text{ of control})$  than in untreated controls

 $(100 \pm 176 \% \text{ of control})$ . Administration of H<sub>2</sub> to alkali-burned animals significantly (\*p < 0.05) reduced phosphorylated NF- $\kappa$ B p65 levels (140 ± 158 % of control) n =28-29. Error bars indicate SD.

**Figure 6** (A) Alkali burn-induced Corneal NV is inhibited with H<sub>2</sub>. NV area in the whole cornea was evaluated semi-quantitatively. Scale bar = 500 µm. NV area was significantly lower (\*\*p < 0.01, B) in H<sub>2</sub> treated mice (27.3 ± 9.0 %) than in vehicle-treated controls (38.1 ± 7.8 %) n =13-15. Corneal VEGF (C) and MCP-1 (D) protein levels were significantly (\*p < 0.05 and \*\*p < 0.01, respectively) higher in vehicle-treated alkali-burned mice (14.9 ± 12.5 pg/mg and 54.4 ± 7.3 pg/mg, respectively) than in vehicle-treated controls (1.9 ± 4.3 pg/mg and non-detectable, respectively). Administration of H<sub>2</sub> to Alkali-burned animals significantly (\*\*p < 0.01 for both) reduced VEGF and MCP-1 protein levels (2.9 ± 2.1 pg/mg and 34.0 ± 21.5 pg/mg, respectively) n = 13-15. Error bars indicate SD.

Supplemental Figure. Deletion of SOD-1 accelerates corneal NV by UVB. (A, B) UVB induced corneal NV in mice cornea. The NV areas in SOD-1<sup>-/-</sup> mice (39.1±7.9%, n = 16) were significantly increased compared with SOD-1<sup>+/+</sup> (22.1±5.6 %, n = 18) (\*\*p< 0.01). Error bars indicate SD. Scale bar = 500 µm.

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Figure 1

24 Kubota



Figure 2

## 25 Kubota Oxidative stress-induced corneal angiogenesis



Figure 3

26 Kubota Oxidative stress-induced corneal angiogenesis



Figure 4



Figure 5

28 Kubota Oxidative stress-induced corneal angiogenesis



Figure 6



Supplementary Figure