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Protective effect of saturated hydrogen saline against blue light-induced retinal damage in rats

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Abstract

AIM

To explore the effect of saturated hydrogen saline on blue light-induced retinal damage in rats.

METHODS

The retinal damage of rats was induced by blue light exposure for 6 hours and examined 8 hours, 16 hours and 24 hours after the exposure. One hundred female Sprague-Dawley rats were randomly divided into four groups. Group 1 included 30 rats received light exposure without any other treatment. Group 2 included 30 rats received light exposure with intraperitoneal injection of normal saline. Group 3 included 30 rats received light exposure with intraperitoneal injection of saturated hydrogen saline. And Group 4 included the other 10 rats which did not receive any treatment. The amount of intraperitoneal injection of saturated hydrogen saline and normal saline was calculated in the ratio of 1ml/100g of rat weight. Specimens were collected and processed by H-E staining, ultrastructure observation, biochemical measurement. Morphological changes were observed by light microscope and transmission electron microscope (TEM) and the retinal outer nuclear layer (ONL) thickness was measured by IPP 6.0, while the malondialdehyde (MDA) was measured by colorimetric determination at 532 nm.

RESULTS

Although the structure of retina in Group 1 and Group 2 was injured heavily, the injury in Group 3 was mild. The differences between Group 1 and Group 2 were not significant. Compared with the rats in Group 1 and Group 2, the ones in Group 3 had more clearly demarcated retina structure and more ordered cells by light microscope and TEM observation. The ONL thicknesses (400 times) of four groups at each time point except between Group 1 and Group 2 were significantly different (P<0.05). The thicknesses of the ONL in Group 1 at three time points were 30.41±4.04μm, 26.11±2.82μm and 20.63±1.06μm, in Group 2 were 31.62±4.54μm, 25.08±3.63μm and 19.07±3.86μm, in Group 3 were 29.75±3.62μm, 28.83±1.97μm and 27.61±1.83μm. In Group 4 the mean of the thickness was 37.35±1.37μm. As time went by, the damage grew more severely. At 24h point, the differences were most significant. Compared with Group 4, the thickness was 46.23% thinner in Group 1, 50.29% thinner in Group 2 and 28.04% thinner in Group 3. The stack structures of membranous disc in Group 3 were injured slightly, but in Group 1 and Group 2 the damage was more obvious by TEM. Compared with Group 4 at each time point, the content of MDA in Group 1 was higher (P<0.05). The content of MDA in Group 3 was significantly lower than those of Group 1 (P<0.05) and Group 2 (P<0.05). Between the Group 1 and Group 2, the MDA concentration at each time point was no significant difference (P>0.05).

CONCLUSION

Saturated hydrogen saline could protect the retina from light-induced damage by attenuating oxidative stress.

Keywords: retina, hydrogen, antioxidants, phototoxicity

INTRODUCTION

A large number of basic researches, clinical trials and epidemiologic studies indicated that long-term exposure to light could induce the formation and accumulation of free radicals and lipid peroxidant, which induced oxidative damages of retinal cells. That results in a range of impairments such as injury, apoptosis, necrosis and cell membrane lysis, leading to apoptosis of photoreceptor cells and retinal degeneration, finally leading to the outbreak of eye disorders, even visual loss. The pathologic mechanism is remarkably similar to the diseases of retinal degeneration such as age-related macular degeneration (ARMD) and retinitis pigmentosa (RP)[1]-[4]. Blue light, as a short wave-length, near ultraviolet spectrum visible light, is the major ray inducing to retinal damage even apoptosis in the natural world. Blue light-induced damage of retinal cells has been widely used as a model for both in vivo and in vitro studies of retinal degeneration[5]-[8]. Clearing redundant free radical and lipid peroxide may prevent and treat retinal light-induced damage for the mechanism[7],[9]. At present, there are lots of reports about the studies of the prevention and treatment of retinal light injury. Hormone, antioxidant, neuroprotectants, cytokine were mentioned in those researches[10]-[14].

Hydrogen is the most abundant chemical element, constituting approximately 90% of the universe's elemental mass. It is a colorless, odorless, nonmetallic, tasteless, highly flammable diatomic gas which is mainly used in fossil fuel processing and ammonia production. During a long period, hydrogen was considered as a physiological inert gas. In 1975, Dole *et al*[15] firstly re-

ported that there was significant cancer regression in patients with squamous cell carcinoma exposed to hyperbaric hydrogen for 2 weeks. In 2001, Gharib *et al*[16] set infected animals stay 2 weeks in a hyperbaric chamber in a normal atmosphere supplemented with 0.7 MPa hydrogen. The treatment had significantly protective effect towards inflammation. In his study, inhalation of hydrogen gas could increase antioxidant enzyme activity and decrease lipid peroxide levels. However, the hyperbaric hydrogen therapy mentioned in these studies was confirmed to be dangerous and difficult in applying in clinical treatments by other researchers. Until Ohsawa *et al*[17] demonstrated that hydrogen gas could be used as a therapeutic mitochondrial antioxidant to neutralize oxidative stress after ischemia-reperfusion injury and then proved hydrogen-dissolved water had the potential to prevent arteriosclerosis[18], hydrogen gas was regarded as a novel and effective treatment. In the past two decades, the studies about hydrogen had come out more and more. As yet, the antioxidation of hydrogen was already reported in almost all disorders related to oxidative stress, such as cancer, ischemia-reperfusion injury, chronic inflammatory disease, type 2 diabetes or impaired glucose tolerance, neurodegenerative diseases like Parkinson's disease and Alzheimer's disease, acute radiation syndrome, noise-induced hearing loss, and so on[15],[16],[19]-[25]. Ohsawa *et al*[17] demonstrated that hydrogen selectively reduced the hydroxyl radical, the most cytotoxic of reactive oxygen species (ROS), and effectively protected cells from acute oxidative stress. Therefore, molecular hydrogen was presumed to be a special free radical scavenger which uniquely reduces hydroxyl radical. In current progress of ophthalmology, it was proved that hydrogen-dissolved saline could treat retinal ischemia-reperfusion injury[26] and hydrogen solution could significantly reduce angiogenesis of cornea after alkali-burn injury[27]. From those researches, hydrogen as an antioxidant can exert anti-oxidation, anti-inflammatory and anti-allergy effect. Thus, treatment with hydrogen has several potential advantages over current therapies used for ischemia-reperfusion or oxidative injury. As above, hydrogen is a potential treatment to prevent and treat many diseases, and can be used in a very wide range of application.

To date, most of these researches have focused on the ischemic and reperfusion injury, but the potential effect of hydrogen in light-induced damage has not been tested. We hypothesize that hydrogen may attenuate light-induced damage by reduction of oxidative stress. In this observation, saturated hydrogen saline was injected into abdominal cavity regularly and light-induced damage was established by exposure to blue light. After dealing with different treatments among groups and the establishment of light-induced damage model, specimens were collected and then measured in several different aspects. Morphological changes were observed by light microscope and transmission electron microscope (TEM) and the retinal outer nuclear layer (ONL) thickness was measured by IPP 6.0, while the malondialdehyde (MDA) was measured by colorimetric determination at 532 nm. The effect and mechanism of saturated hydrogen saline to the retina from light-induced damage was investigated.

MATERIALS AND METHODS

Materials

Reagents Coomassie brilliant blue kit and MDA kit were supplied by Nanjing Jiancheng Bioengineering Institute, China. Hydrogen gas was supplied by The Oxgen Company WISCO, Ltd. Other reagents were analytical reagents.

Animals and groups A hundred healthy female rats weighing 160g-180g were randomly assigned to the following four groups: 1) Group 1 (exposure to light only, *n*=30), 2) Group 2 (light damage and normal saline treatment, *n*=30), 3) Group 3 (light damage and saturated hydrogen saline treatment, *n*=30), 4) normal group (without any treatment, *n*=10). The eyeballs were collected 8 hours, 16 hours and 24 hours after withdrawal to light in anterior 3 groups. The eyeballs in Group 4 were collected as negative control. At each time point, 5 rats were sacrificed for morphological measurement, another 5 rats for biochemical measurement. The rats were supplied by Tongji Medical College Experimental Animal Center, Huazhong University of Science and Technology. They were maintained in 12 h: 12h light: dark cycle of 110lux and free access to food and water ad libitum. Before exposure to light, rats were set in darkness 36 hours for adaption. Treatment of the animals was in conformity with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Saturated hydrogen saline production Saturated hydrogen saline were prepared by bubbling H_2 gas (flow rate:1 L/min) through 400ml of normal saline solution with stirring for 10min to a saturated level, and stored under atmospheric pressure in an aluminum bag with no dead volume[26]. The hydrogen content was confirmed with a hydrogen electrode. The half-life of the saturated hydrogen saline is approximately 3 months. The saturated hydrogen saline was freshly prepared every week, which ensured that the concentration of more than 0.6 mmol/L was maintained.

Exposure to light[28] A cage of 1m in length, 1m in width, 0.5m in height was made for the experiment. There were 5 blue fluorescent lamps setting on the inside surfaces of the cage (expect the bottom). The rats were exposed to 400-480nm light for 6 hours after 36-hour darkness adaptation. The blue light emitted continuously at an irradiance of 0.64w/m^2. The temperature in the cage was 24±2°C. After dilated pupils by 1% atropine, rats were intraperitoneally anesthetized with chloral hydrate (300mg/kg). Nylon wires were used to keep the rats' eyes open during the exposure session. After waking up, rats were put into the cage where the lamps preheated. Transparent glass plates were used to separate each rat in order to avoid the rats hiding from the light. Immediately after 6 hours of continuous light exposure, rats were got the sutures out and placed in darkness.

Methods

Medication Before and during the exposure session, the rats in Group 2 and Group 3 were injected normal saline or saturated hydrogen saline 1ml/100g of rat weight once a day[29], respectively. For Group 1, rats were just exposure to light without any treatment. Rats in Group 4 received no light exposure or treatment. The whole process of the experiment lasted 21 days.

Hematoxylin-eosin (HE) staining Five rats were anesthetized at each time point with chloral hydrate (300mg/kg), and the eyes were immediately enucleated. The left eyes were fixed in 4% paraformaldehyde for 20-22hours. Paraffin-embedded sections of 5μm were cut in the sagittal plane and stained with H-E kit. The structure of retina was observed by light microscope and the thickness of outer nuclear layer (ONL) was measured by IPP 6.0.

Transmission electron microscope (TEM) examination After putting the left eyes into 4% paraformaldehyde, the right eyes were immediately enucleated and fixed in 3% glutaraldehyde overnight. The upper temporal regions 1-2mm from the optic nerve head were soaked in 1% osmium tetroxide. Following serial dehydration, the tissue was stained with uranyl acetate, and embedded in Agar 100. Sections of 0.1μm from retina were prepared. We observed under transmission electron microscope and took photographs.

Determination of MDA concentration in retina Retinal MDA concentration, a presumptive marker of oxidative product of lipid peroxidation, was quantified to estimate the extent of lipid peroxidation in the light-induced damage. The retinas, separated from 5 eyecups, were homogenized at 10% in buffer for 10-15 minutes, then centrifugated at 3000r/min at 4°C for 15 minutes. After determining protein concentration, the MDA concentration was measured by the method of colorimetry and expressed as nmol/mg. The saline was used for the standard curve.

Statistical Analysis

All quantitative date are expressed as means±SD. For comparison the changes of the thicknesses of ONL and concentration of MDA, differences between groups were determined with a one-way ANOVA followed by Student-Newman-Keuls tests. $P<0.05$ was considered to denote statistical significance.

RESULTS

Morphological Measurements by H-E staining

Blinded histological analysis of eyeball sections stained with H-E were scored 8 hours, 16 hours, 24 hours after light exposure (Figure 1). More dead cells appeared in Group 1 and Group 2 after light damage. In Group 1 and Group 2, cells arranged sparsely and the cell outline was fuzzy. In Group 3 and Group 4, the cell outline was clear and the structure was compact. Cells were big and have abundant cytoplasm. Retinas in Group 3 had clearly demarcated structure and more ordered cells compared with retinas in Group 1 (Figure 1G, H, I). Long-term exposure to light decreased the thickness of ONL. The thickness of ONL was greatly reduced at 24h after light exposure (Figure 1C, F, I).Quantitatively morphometric analysis of ONL thickness in retina at each time point shows in Figure 2.

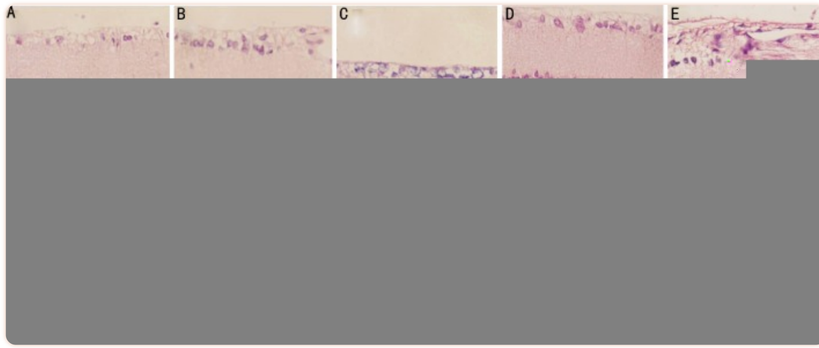

Figure 1

H-E analysis of retinal sections after exposure to blue light for 6 hours

The specimens were collected 8 hours (A, D, G), 16 hours (B, E, H) or 24 hours (C, F, I) after withdrawal of the light. A, B, C are the photographs for Group 1, D, E, F are the photographs for Group 2 and G, H, I are photographs for Group 3. J is a photograph for Group 4.

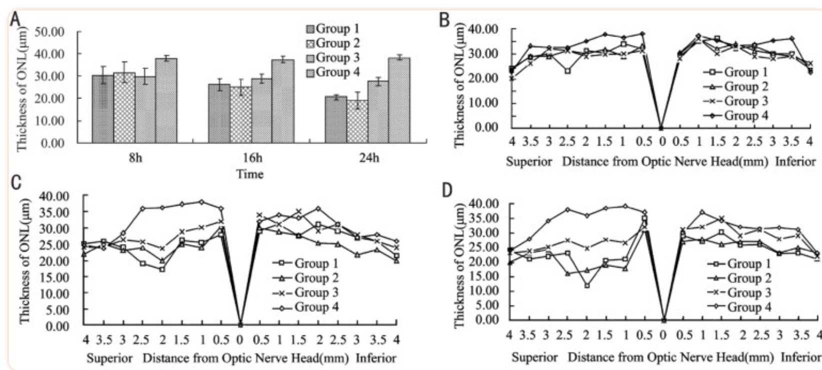

Figure 2

Quantitative illustration of thickness of ONL

A: The mean of the thickness of ONL in the region 1-2mm from the optic nerve head within the superior hemisphere was derived from five sections of five rats at each time point, differences were significant after blue light exposure at each time point, with compared incidence (P<0.05); B, C D: The composite of date of ONL thickness in retinas at different time points. All data were the means for rats followed different treatments and exposed to light 8, 16 and 24 hours after withdrawal of the light.

Ultrastructure examination by TEM

By ultrastructure examination, the changes of retina were significantly different at 16 hours after withdrawal of the light (Figure 3). The nuclei of dying cells were usually shrunken and round in sharp. Degenerating cells appeared among the ONL. Cytoplasm and chromatin condensation, nu-

clear fragmentation could be seen in the affected cells. In Group 1 and Group 2, the mitochondria were inflated and the ridges were disrupted. The outer segments were vesiculate and disrupted. The membrane disks in Group 1 and Group 2 were broken severely. Occasionally, the apoptotic body was visible. Additionally, mild swelling of the mitochondria could be seen in the inner segment in Group 3. However, no inflammatory response was evident in section subjected to inspection.

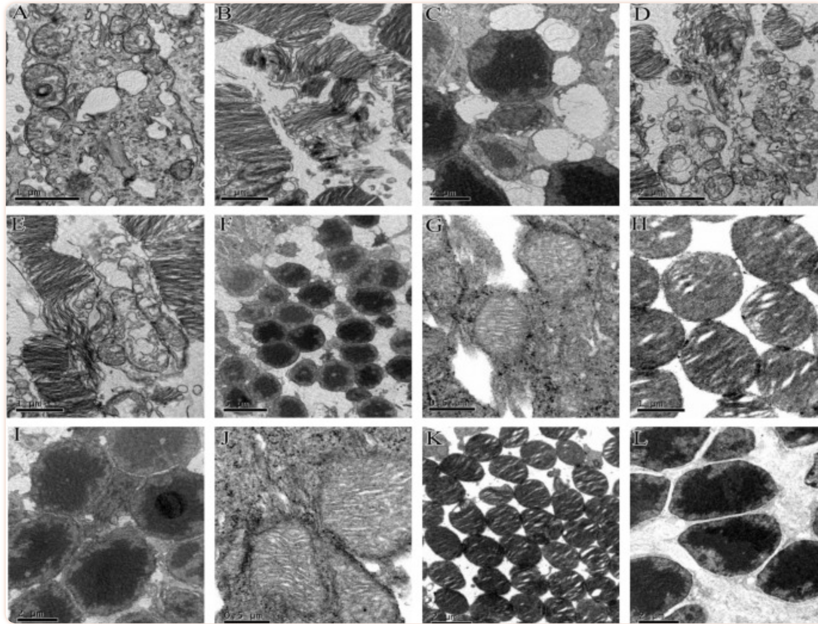

Figure 3

Transmission electron micrographs

A, B, C: Photographs for Group 1; D, E, F: Photographs for Group 2; G, H, I: Photographs for Group 3; J, K, L: Photographs for Group 4.

Retinal MDA concentration after exposure

The MDA concentration was measured at 8h, 16h, and 24h after withdrawal of the light (Table 1). Retinal MDA concentration at 16h in Group 3 was significantly lower than that of Group 1. Between groups at the same time point, the differences were conspicuous expect between Group 1 and Group 2 (P<0.05).

Table 1

Quantitative date of MDA concentration in rats' retinas at different time point

Measures	MDA(nmol/mg)		
	8h(n=5)	16h(n=5)	24h(n=5)
Group 1	55.72±3.94[a]	69.23±5.0[a]	80.16±4.91[a]
Group 2	56.21±3.53[a,b]	72.89±3.83[a,b]	79.98±6.36[a,b]
Group 3	49.59±4.15[a,c]	57.26±6.34[a,c]	69.01±4.11[a,c]
Group 4		43.48±4.05	

Data are expressed as mean±*s*. Lower values indicate better function of clearing lipid peroxidant.

One-way ANOVA test was used to compare values between two groups at the same time points. a means compared with Group 4, b means compared with Group 1 and c means compared with Group 1. The superscripts a and c showed *P* values less than 0.05, and b denoted *P* values greater than 0.05.

DISCUSSION

As we are aware, this is the first study demonstrating that saturated hydrogen saline alleviated the light-induced damage of rats' retinas. The improvement was paralleled to a significant protection of cells and organelles, attenuation of retinal apoptosis in ONL, decreased retinal MDA concentration. This improvement may result from radical oxygen species (ROS) scavenging effect of molecular hydrogen, as previously reported in other injury models[19],[23],[30].

Taken together a large amount of work studied about light damage, we now know that retinal light damage is a multi-factorial process involving both environmental and genetic factors. At present, three light-induced damage hypotheses, originally presented in Noell's landmark publication, were generally objected by scholars, and have guided much of the work in this area. These hypotheses include: a toxic photoproduct arising from vitamin A during exposure to intense light; a metabolic abnormality resulting from light exposure; and light-induced oxidative reactions[31]. Many scholars did a lot of additional work, approached the mechanism of light damage and the way to prevent and treat the retinal light damage for the therapy of human retinal disease about retinal degeneration[10],[11].

The effect of light-induced damage on retinal morphology can be dramatic, because the entire population of visual cells is often adversely affected. Light-induced damage is typically confined to rod photoreceptors and retinal cells loss restricted to the outer nuclear layer (ONL)[32],[33]. Thus, measurements of thickness of ONL can be used to quantify light-induced damage. Light-induced damage in retina is a graded response, with areas containing little or no damage adjacent to more

severely affected. Rapp and Williams[34] found that, within the superior hemisphere, light-induced damage was often particularly severe in a region 1-2 mm from the optic nerve head. So, we took the temporal region there to observe the ultrastructure.

Retina is susceptible to oxidative stress due to the high levels of polyunsaturated fatty acids, photosensitizers and pigments and the high consumption of oxygen. In addition, it is compelling evidence showing that exposure to visible light creates conditions of photo-oxidative stress, leading to oxidative damage of photoreceptors[35],[36]. Free radical produces lipid peroxidation, protein peroxidation, DNA peroxidation and oxidative stress which are the central elements of age-related diseases[37]. Clinical and basic science evidences indicate that oxidative stress is a common contributor to many degenerative diseases, including retinal disorders such as age-related macular degeneration (ARMD)[2],[4],[9],[35]. Therefore, in this study we measured the levels of the malondialdehyde (MDA) which is the product of lipid membrane oxidation and could represent the production of the reactive oxygen.

It was demonstrated that hydrogen selectively reduces the hydroxyl radical, the most cytotoxic of reactive oxygen species (ROS), and effectively protects cells[17]. Hydrogen molecule is electrically neutral and small, so it easily penetrates membranes and enters into the nucleus and mitochondria. It can react with cytotoxic ROS and thus protect cells against oxidative damage. It is possible that the intracellular hydrogen concentration increases immediately following injection of saturated hydrogen saline and lasted at a high level by continual application. Since the retina is a high oxygen consumption tissue, immediately clearing the oxidant is necessary. In the present study, we analyzed the mechanism of saturated hydrogen saline in a rat model of exposure to blue light, and observed that saturated hydrogen saline treatment actually decreased oxidative stress. The major findings of the study were that saturated hydrogen saline significantly protected the retinal cells from the light-induced damage, suppressed the early accumulation of lipid peroxidation products. Compared with Group 1 and Group 2, the thickness of ONL in Group 3 was thicker, and the morphologic changes were slighter, although it was still significantly different with Group 4. At each time point, we found reduced MDA concentration in Group 3. Consistent with previous reports, in the present study we found treatment with saturated hydrogen saline protected retina by reducing oxidative stress in light-induced damage. Previously, other therapies for scavenging reactive oxygen species seemed promising in animal models but most of them failed in clinical trials[38]. On the base of our observation and other previously reports, saturated hydrogen saline which is safe, economic and easy to manufacture, could be a novel therapeutic tool for future retinal protection. Our finding may open new avenues for preventing light-induced damage associated with ARMD and RP.

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