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Research article

Protective effects of hydrogen-rich saline against N-methyl-N-nitrosourea-induced photoreceptor degeneration

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ABSTRACT

The N-methyl-N-nitrosourea (MNU)-treated rat is typically used as an animal model of chemically-induced retinitis pigmentosa (RP). Reactive oxygen species (ROS) have been recognized as the crucial contributor to the retinal photoreceptor apoptosis seen in MNU-treated rats. In the present study, we explored the therapeutic effects of hydrogen-rich saline (HRS), a selective ROS scavenger, on MNU-induced photoreceptor degeneration. Intraperitoneal (IP) administration of HRS ameliorated MNU-induced photoreceptor degeneration in terms of morphology and function: Sharply decreased thickness of the retinal outer nuclear layer (ONL) and flattened photopic and scotopic electroretinogram (ERG) waveforms, typically seen in response to MNU treatment, were substantially rescued in rats cotreated with MNU and HRS (MNU + HRS). Moreover, the terminal deoxyuridine triphosphate nick-end labeling (TUNEL) assay revealed a smaller number of apoptotic photoreceptors in the MNU + HRS group compared that in the MNU group. Compared to MNU-treated rats, retinal malondialdehyde (MDA) content in MNU + HRS rats significantly decreased while superoxide dismutase (SOD) activity significantly increased. Morphological and multi-electrode array (MEA) analyses revealed more efficient preservation of the architecture and field potential waveforms in particularly the peripheral regions of the retinas within the MNU + HRS group, compared to that in the MNU group. However, this enhanced protection of structure and function in the peripheral retina is unlikely the result of site-dependent variation in the efficacy of HRS; rather, it is most likely due to reduced susceptibility of peripheral photoreceptors to MNU-induced degeneration. Inner retinal neuron function in the MNU + HRS rats was better preserved, with fewer apoptotic photoreceptors in the ONL. Collectively, these results support the rationale for future clinical evaluation of HRS as a therapeutic agent for human RP.

1. Introduction

Retinitis pigmentosa (RP) is a heterogeneous group of inherited retinal dystrophies that is characterized by progressive photoreceptor apoptosis. The prevalence of RP is reported to be approximately 1 in 4000 individuals in western countries, with more than 1.5 million patients suffering from the progressive visual deterioration. Current small scale clinical trials or experimental therapies against RP include gene therapy, neuroprotection, anti-apoptotic agents, retinal transplantation, retinal prostheses, and stem cell therapy (Hartong et al., 2006). However, due to the complexity of RP pathogenesis, the overall prognosis of RP remains dismal. Thus far, nutritional therapy with vitamin A (15,000 IU/day) is the only established clinical treatment for retarding RP, and the therapeutic effect is far from satisfactory. Reactive oxygen species (ROS) have been recognized as the crucial contributor to photoreceptor apoptosis in RP (Carmody and Cotter, 2000; Komeima et al., 2008; Yu et al., 2004, Yu and Cringle, 2005). ROS subtypes, such as hydroxyl (OH^-) and peroxynitrite ($ONOO^-$) radicals, can induce mitochondrial DNA stress and lipid oxidation, leading to mitochondrial membrane breakdown and release of cytochrome-c, which further promotes activation of the apoptotic cascade. It has

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been found that ROS excessively elevates poly ADP-ribose polymerase activity and triggers photoreceptor apoptosis via its interaction with transcription factors, such as nuclear factor-κB and activator protein-1 (Shen et al., 2005). Consequently, it is important for the antioxidant defense system to neutralize these cytotoxic ROS; otherwise, they will interact with macromolecules, including unsaturated lipids, proteins, deoxyribonucleic acids, and iron, which are essential for photoreceptor survival (Lohr et al., 2006; Mizukoshi et al., 2010).

Hydrogen, a colorless, tasteless, odorless, and highly flammable diatomic gas, was initially recognized as a medical therapeutic agent in 1975 (Dole et al., 1975). More recently, Ohsawa et al. (2007) demonstrated the protective effects of hydrogen in a rat model of cerebral infarction and proposed a novel important role of hydrogen as a gaseous radical scavenger. Thereafter, the therapeutic effects of hydrogen have been reported for a wide range of diseases, including transplantation-induced intestinal graft injuries; ischemia-reperfusion injury in the brain, liver, myocardium, intestine, and kidney; cognitive deficits; inflammatory diseases related to oxidative stress; Parkinson's disease; and metabolic syndromes (Fukuda et al., 2007; Kajiya et al., 2009; Sun et al., 2009; Cardinal et al., 2010; Chen et al., 2010; Li et al., 2010; Nakao et al., 2010; Ono et al., 2011). Hydrogen can exert therapeutic antioxidant and antiapoptotic effects via selectively targeting the cytotoxic ROS, such as the OH^- and $ONOO^-$ radicals. In contrast, hydrogen is far less effective against the functional ROS, such as superoxide and hydrogen peroxide, which play physiological roles in the upregulation of endogenous antioxidant enzymes (Ohsawa et al., 2007; Feng et al., 2013). An additional advantage of hydrogen is that it exerts no cytotoxicity, even at high concentrations. Moreover, safety standards have been established for high concentrations of inhaled hydrogen gas, as high-pressure hydrogen is used in deep diving gas mixes to prevent decompression sickness and arterial gas thrombi (Abraini et al., 1994). Hydrogen can be dissolved in water up to 0.8 mM under atmospheric pressure at room temperature. A solubilized form of hydrogen, known as hydrogen-rich saline (HRS), is advantageous because it is a safe, portable, and easy approach for therapeutic hydrogen delivery (Zhou et al., 2013; Zhang et al., 2014).

Both *in vivo* and *in vitro* studies have verified the antioxidant properties of HRS. In a pioneering study, it was found that HRS treatment can retard cataract formation and restore antioxidant capacity in a selenite cataract model via maintaining the activities of multiple endogenous antioxidant enzymes, such as the SOD, glutathione peroxidase, glutathione reductase, and glutathione transferase (Yang et al., 2013). With respect to retinal disease, investigators have found that HRS can protect the retina from light-induced damage via the sirtuin type 1 signaling pathway. These investigators also found that HRS can increase the expression of antiapoptotic factor Bcl-2 and the activity of SOD, thereby ameliorating the apoptosis and oxidative stress in the light-damaged retinas (Qi et al., 2015).

Rats treated with N-methyl-N-nitrosourea (MNU) to chemically induce photoreceptor degeneration have been widely used as a model of human RP (Tsubura et al., 2011). ROS is considered as a key contributor to the pathogenesis of MNU-induced photoreceptor degeneration (Hebels et al., 2010). Moreover, with the help of multi-electrode array (MEA) recording, MNU-induced alterations in inner retinal neurons can be examined before and after HRS treatment (Tao et al., 2015a). Based on the afore mentioned findings, we used MEA recording and other methods to characterize the effects of HRS against photoreceptor degeneration in the rodent RP model, thereby allowing us to evaluate the potential of HRS as an effective therapeutic agent for human RP.

2. Methods

2.1. Animals and MNU administration

All experiments were conducted in accordance with the ARVO Statements for the Use of Animals in Ophthalmic and Vision Research. Every effort was made to minimize the number of animals used and their suffering. All procedures regarding the use and handling of the animals were conducted in accordance with the Institutional Animal Care and Use Committee of the Fourth Military Medical University. For this study, Sprague-Dawley rats of both sexes (8–9 weeks old) were maintained under standard laboratory conditions (room temperature of 18–23 °C, 40–65% humidity, 12 h dark/light cycle), with food and water available *ad libitum*. MNU (Sigma-Aldrich, St. Louis, MO, USA) was stored in the dark at −20 °C, and it was dissolved in physiological saline containing 0.05% acetic acid just before use. Rats received an intraperitoneal (IP) injection of MNU at a dose of 60 mg/kg. No deaths occurred, and no clinical signs of toxicity in any of the MNU-treated animals were evident during the experiment. Functional and morphological examinations were performed at time points of 3 days (P3) and 7 days (P7) post MNU injection.

2.2. HRS preparation and administration

HRS was prepared according to the method previously described (Tian et al., 2013). Briefly, hydrogen was dissolved in physiological saline for 6 h under high pressure (0.4 MPa) to achieve a supersaturated solution. Under atmospheric pressure at 4 °C, the HRS was stored in an aluminum bag without dead volume. To maintain the concentration above 0.6 mmol/L, HRS was freshly prepared every week. Hydrogen content in the saline was confirmed using a previously described gas chromatographic method (Ohsawa et al., 2007). A daily IP dose of HRS (10 ml/kg) was administered from 14 days prior to MNU administration until sacrifice. Another group of rats received the same volume of only physiological saline during the same time period, and they were used as the control for the hydrogen and MNU treatments.

2.3. Electroretinogram (ERG) recording

The ERG recordings were performed according to a previously described method (Tao et al., 2013). Briefly, animals were dark adapted overnight (>12 h) and prepared for recording under dim red light. Anesthesia was induced with IP injections of 1% sodium pentobarbital (3 ml/kg, Sigma-Aldrich) and Sumianxin II—a compound preparation of xylidinothiazoline, EDTA, dihydroetorphine hydrochloride and haloperidol (0.025 ml/kg, Jilin Shengda Animal Pharmaceutical Co., Ltd., Jilin, China). The anesthetized animals were then placed on a heating pad throughout the recording session. Corneal anesthesia was achieved with one drop of proxymetacaine (0.5%), and each cornea was kept moist with physiological saline. The pupils were dilated to a diameter of about 5 mm with compound tropicamide eye drops (tropicamide, 5 mg/ml; phenylephrine hydrochloride, 5 mg/ml).

Full-field ERGs were recorded using the RETI-port system (Roland Consult, Havel, Germany) with custom-made silver chloride electrodes. The active electrode was a ring electrode placed at the center of the cornea. Stainless steel needle electrodes were placed in the cheek and tail to serve as the reference and ground leads, respectively. The stimulus was a brief white flash (3.0 cd-s/m^2) delivered via a Ganzfeld integrating sphere. Signals were amplified and filtered to a bandpass of 1–300 Hz. A 50-Hz notch filter was applied to eliminate line noise. A total of 60 photopic responses and 10 scotopic responses were recorded and averaged