Inhalation of hydrogen gas attenuates cisplatin-induced ototoxicity via reducing oxidative stress

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ARTICLE INFO

Article history:
Received 26 August 2011
Received in revised form 12 October 2011
Accepted 13 October 2011
Available online 3 November 2011

Keywords:
Cisplatin
Oxidative
Hearing loss
Hydrogen gas (H2)

ABSTRACT

Objective: Cisplatin, an anticancer drug used extensively to treat a broad range of tumors, has strong ototoxic side effects induced by reactive oxygen species (ROS). Recently, it has been reported that hydrogen gas (H2) is a new antioxidant by selectively reducing hydroxyl radical, the most cytotoxic ROS. The present study was designed to investigate whether H2 treatment is beneficial to cisplatin-induced ototoxicity via reducing oxidative stress.

Methods: The animals were intraperitoneally given a 30 min infusion of 16 mg/kg cisplatin or the same volume of saline. H2 treatment was given twice with 2% H2 inhalation for 20 min starting at 1 h and 6 h after cisplatin or saline injection, respectively. The hearing status of all animals was assessed by auditory brainstem responses (ABR). The hair cell damage was observed by phalloidin staining. In addition, the levels of oxidative products in serum and cochlear tissue were measured.

Results: We found that H2 treatment significantly attenuated cisplatin-induced hearing loss evaluated by click-evoked and tone burst ABR threshold. Furthermore, histological analysis revealed that 2% H2 treatment significantly alleviated cisplatin-induced hair cell damage in the organ of Corti. In addition, cisplatin significantly increased the levels of malondialdehyde (MDA) and 8-iso-prostaglandin F2α (8-iso-PGF2α) in serum and cochlear tissue, which was attenuated by H2 treatment.

Conclusion: These results demonstrate that H2 is beneficial to cisplatin-induced ototoxicity via reducing oxidative stress. Therefore, H2 has potential for improving the quality of life of patients during chemotherapy by efficiently mitigating the cisplatin ototoxicity.

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1. Introduction

Cisplatin (cis-diamine-dichloroplatinum II) is currently one of the most effective chemotherapeutic agents, which can be widely used in the treatment of a variety of tumors, such as those of head, neck, testis, ovary and breast [1,2]. Unfortunately, irreversible ototoxicity is a serious side effect of cisplatin treatment, which leads to hearing loss in approximately half a million new cancer patients annually in the United States [3]. The hearing loss is due, in part, to the increased generation of reactive oxygen species (ROS) in cochlea, leading to lipid peroxidation and damage or death of outer hair cells in the organ of corti [4,5]. Antioxidant therapy might be clinically useful in preserving hearing, but there is evidence that antioxidants may interfere with the tumoricidal action of cisplatin [5]. Currently, there are no effective treatments against cisplatin-induced ototoxicity in clinical use.

In 2007, Ohsawa et al. has found that hydrogen gas (H2) exerts a therapeutic antioxidant activity by selectively reducing hydroxyl radicals (*OH, the most cytotoxic ROS) without interfering with other physiological ROS [6]. Recent studies have shown that H2 has widely therapeutic roles in many diseases via reducing oxidative stress, inflammation and apoptosis, such as ischemia–reperfusion injury, sepsis, multiple organ dysfunction, acute pancreatitis, chronic allograft nephropathy, tumor and type 2 diabetes [6–15]. Furthermore, H2 can effectively protect auditory hair cell against the morphological and functional damage induced by ROS [16]. More importantly, pretreatment with H2-rich water can facilitate the recovery of hair cell function and attenuate noise-induced temporary hearing loss [17]. In addition, H2 alleviates nephrotoxicity induced by cisplatin without compromising anti-tumor activity in mice [18]. These findings strongly indicate that H2 treatment may be beneficial to cisplatin-induced ototoxicity.
In the present study, therefore, we tested the hypothesis that H₂ could attenuate cisplatin-induced ototoxicity and hearing loss in rats via reducing oxidative stress.

2. Materials and methods

2.1. Animals

Male Wistar rats of 250–300 g with normal Preyer's reflex were used in the study. The animals were provided by the animal center of the Fourth Military Medical University in China. The procedures in this study were approved by the Animal Care and Use Committee at the Fourth Military Medical University.

2.2. Grouping

The animals were randomized to one of four groups (n = 6 per group): saline, saline + H₂, cisplatin and cisplatin + H₂. The animals in the cisplatin and cisplatin + H₂ groups were intraperitoneally given a 30 min infusion of 16 mg/kg cisplatin (Sigma–Aldrich Corp., St. Louis, MO, USA) [19]. The remaining two groups received saline (isotonic sodium chloride solution) injection in equal volume. Based on our previous studies and preliminary experiment [11,12,14,15], H₂ treatment was given twice with 2% H₂ inhalation for 60 min starting at 1 h and 6 h after cisplatin or saline injection, respectively.

2.3. H₂ treatment

The animals with H₂ treatment were put in a sealed plexiglas chamber with inflow and outflow outlets. H₂ was supplied through a gas flowmeter, TF-I (YUTAKA Engineering Corp., Tokyo, Japan), and delivered by air into the chamber at a rate of 4 L/min. The concentration of H₂ in the chamber was continuously monitored with a commercially available detector (Hy Alerta Handheld Detector Model 500, H₂ Scan, Valencia, CA, USA). The concentration of oxygen in the chamber was maintained at 21% by using supplemental oxygen and continuously monitored with a gas analyzer (Medical Gas Analyzer LB-2, Model 40M, Beckman, USA). Carbon dioxide was removed with baralyme. The animals without H₂ treatment were exposed to room air in this chamber [11,12,14,15].

2.4. Auditory brainstem responses

To investigate the effects of H₂ on cisplatin-induced otoxicity, the hearing status of all animals was evaluated at baseline just before and again 3 d after cisplatin or saline administration using auditory brainstem responses (ABR) as previously described [17,20]. Elevation of ABR threshold has been shown to provide an excellent, reliable indicator of the degree of cochlear hearing loss in experimental animals [21]. Animals were anesthetized with an intraperitoneal injection of 40 mg/kg pentobarbital sodium. The needle electrodes were placed subcutaneously beneath the pinna of measured ear (reference electrode), the apex of nose (ground), and the vertex (active electrode). The stimulus signal was generated through an Intelligent Hearing Systems device (Bio-Logic Systems, USA). Click sounds at a rate of 57.7/s were given to evoke the ABRs. Tone burst sounds at 4, 8, 16 and 32 kHz (0.2-ms rise/fall time and 1-ms flat segment) were generated to estimate frequency-specific thresholds. Responses of 1024 sweeps were averaged at each intensity level step. The stimulus intensity varied in 5 dB sound pressure levels (SPL) stepwise increments, and the threshold was defined as the lowest intensity level at which a response was still observed. Rectal temperature was monitored and maintained at 37 °C by a warming pad during the experiment.

2.5. Detection of hair cell damage

To observe the hair cell damage [22], all animals were sacrificed by decapitation under deep anesthesia after the last ABR test (at 3 d after cisplatin or saline injection). The cochlea was quickly removed and fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 24 h at room temperature. After fixation, the cochleae were dissected. After permeabilization with 0.3% Triton X-100 in PBS for 10 min, the organ of corti was stained for filamentous actin with Alexa Fluor 488 conjugated to phalloidin (green fluorescence) (Molecular Probes, Eugene, OR, USA) for 40 min to outline hair cells and their stereocilia for a quantitative assessment. Hair cell loss was evaluated at each cochlear turn with a fluorescence microscope (Nikon Eclipse 80i, Japan).

2.6. Detection of oxidative products

To investigate the mechanism, the levels of oxidative products in serum and cochlear tissue were measured after the last ABR test (at 3 d after cisplatin or saline injection). The levels of malondialdehyde (MDA) and 8-iso-prostaglandin F₂α (8-iso-PGF₂α) were detected by commercial kits (Cayman Chemical Company, Ann Arbor, MI, USA) using a microplate reader (CA 94089, Molecular Devices, Sunnyvale, Canada). The concentration of tissue protein was determined by using a standard commercial kit (Sigma–Aldrich Corp., St. Louis, MO, USA).

2.7. Statistical analysis

All data are expressed as mean ± SEM. The data were analyzed by one-way ANOVA followed by LSD-t Test for multiple comparisons. The statistical analysis was performed with SPSS 16.0 software. In all tests, a P value of less than 0.05 was considered statistically significant.

3. Results

3.1. H₂ treatment attenuated cisplatin-induced hearing loss

The hearing status was evaluated before and 3 d after cisplatin or saline injection by click-evoked and tone burst ABR threshold shift shown in Figs. 1 and 2. In the cisplatin group, there was one mouse died on day 2 and 83.3% of mice survived during the experiment. In contrast, all mice survived in the cisplatin + H₂ group. Intraperitoneal injection of 16 mg/kg cisplatin significantly increased the click-evoked and tone burst ABR threshold at 4, 8, 16 and 32 kHz when compared with saline injection group (P < 0.05, n = 6 per group), suggesting cisplatin caused significant hearing loss. However, H₂ treatment markedly attenuated the click-evoked and tone burst ABR threshold shift at 4, 8, 16 and 32 kHz in cisplatin-challenged rats (P < 0.05, n = 6 per group). In addition, H₂ treatment had no effects on the click-evoked and tone burst ABR threshold in the rats with saline injection (P > 0.05, n = 6 per group). The results suggest that cisplatin causes hearing loss in rats, which can be significantly attenuated by 2% H₂ treatment.

3.2. H₂ treatment attenuated cisplatin-induced damage of hair cells

Furthermore, because cisplatin mainly damages outer hair cells (OHCs) [3,4], the representative micrographs of hair cell staining in basilar turn were shown in Fig. 3. Cisplatin significantly caused the damage of OHCs, which was improved by H₂ treatment. In addition, the lost OHCs in the basal, second, third and apical turns were counted (Fig. 4). H₂ treatment significantly alleviated cisplatin-induced OHCs loss (P < 0.05, n = 6 per group). The results
suggest that cisplatin causes hair cell damage in rats, which can be significantly attenuated by 2% H₂ treatment.

3.3. H₂ treatment attenuated cisplatin-induced oxidative stress in serum and cochlear tissue

To investigate the underlying mechanism, we detected the levels of MDA and 8-iso-PGF2α in serum and cochlear tissue after the last ABR test (at 3 d after cisplatin or saline injection) (Fig. 5). Cisplatin led to the increased levels of MDA and 8-iso-PGF2α in serum and cochlear tissue when compared to that of saline group (P < 0.05, n = 6 per group). However, H₂ treatment significantly decreased the levels of MDA and 8-iso-PGF2α in serum and cochlear tissue in cisplatin-challenged rats (P < 0.05, n = 6 per group). In addition, H₂ treatment had no effects on the levels of MDA and 8-iso-PGF2α in serum and cochlear tissue in the rats with saline injection (P > 0.05, n = 6 per group). The results suggest that cisplatin results in significant oxidative stress, which can be attenuated by 2% H₂ treatment.

4. Discussion

In the present study, we have found that 2% H₂ treatment improved the click-evoked and tone burst ABR threshold shift induced by intraperitoneal injection of cisplatin. Furthermore, H₂ treatment attenuated cisplatin-induced hair cell damage in rats.

In addition, H₂ treatment decreased the levels of MDA and 8-iso-PGF2α in serum and cochlear tissue in cisplatin-challenged rats. These results demonstrate that H₂ treatment may be a useful therapeutic agent for cisplatin-induced ototoxicity via reducing oxidative stress.

For systemic cisplatin administration, the Wistar rat is a well established model of ototoxicity [23]. Unlike the chinchilla, the Wistar rats have good renal clearance and cisplatin ototoxicity can be induced without high mortality [23]. When compared with multiple doses of cisplatin, a single high-dose administration resulted in a similar pattern of hearing loss that occurred more rapidly and remained stable for at least 6 d [23-24]. In this study, we found that intraperitoneal administration of 16 mg/kg cisplatin caused significant hearing loss evaluated by ABR threshold shift and outer hair cell damage in Wistar rats, which is consistent with previous studies [23,24].

Administration of platinum containing drugs, such as cisplatin, causes significant hearing loss, which is usually permanent and cumulative [4,5]. The organ of corti represents a major site for cisplatin-induced hearing loss, where the drug leads to the permanent loss of outer hair cells [3,4]. Several reports have concluded that the generation of ROS is linked to cisplatin ototoxicity [3-5]. Antioxidant therapy has proven to be beneficial in animal models of cisplatin ototoxicity, but there is evidence that antioxidants may interfere with the tumoricidal action of cisplatin [5]. Therefore, new or improved methods are needed for...
alleviating the cisplatin ototoxicity without compromising its anticancer effect. H₂ can selectively alleviate hydroxyl radicals and peroxynitrite radical-induced cytotoxicity without affecting other ROS, such as superoxide, hydrogen peroxide, or nitric oxide [6]. It is advantageous in medical treatments because the use of hydrogen could not cause serious unwanted side effects. We and other researchers have found that H₂ treatment can effectively protect against organ damage such as brain, spinal cord, heart, lung, liver and kidney through reducing oxidative stress, suggesting that H₂ has a potential role in preventive and therapeutic applications for organ damage [6–15,25]. Furthermore, an in vitro study has also demonstrated the potential of hydrogen to protect both the inner hair cells and outer hair cells from oxidative damage induced by different concentrations of antimycin A [16]. Incubation with a hydrogen-saturated medium significantly reduced ROS generation and subsequent lipid peroxidation in the auditory epithelia, leading to the increased survival of hair cells [16]. Recently, a study from our department has shown that pretreatment with H₂-rich water can prevent noise-induced hearing loss [17]. In addition, H₂ has antitumor effects [9]. H₂ treatment can alleviate nephrotoxic side effects induced by cisplatin without compromising its antitumor activity [18,26]. These studies supported that H₂ may be a good choice for treating cisplatin-induced ototoxicity. In the present study, we found that 2% H₂ treatment significantly attenuated the cisplatin-induced hearing loss evaluated by click-evoked and tone burst ABR threshold. In addition, H₂ treatment could significantly mitigate the hair cell damage induced by cisplatin. These results demonstrate that H₂ is beneficial to cisplatin-induced ototoxicity.

MDA is a commonly measured end point of free radical-induced lipid peroxidation, and the MDA level correlates with the extent of free radical-induced damage [14,27]. In addition, measurement of 8-iso-PGF2α, free radical-catalyzed products of arachidonic acid, can offer a reliable approach for quantitative measurement of oxidative stress status in vivo [28]. Thus, the detection of MDA and 8-iso-PGF2α has been widely used to estimate the overall status of oxidative stress. In the present study, we found that cisplatin significantly increased the levels of MDA and 8-iso-PGF2α in serum and cochlear tissue, which was attenuated by H₂ treatment. This suggests that the decrease of oxidative stress may contribute to the protection of H₂ treatment, which is consistent with our previous studies [11,12,14,15].

In conclusion, the present study supports that H₂ inhalation may be an effective therapeutic agent attenuating cisplatin-induced ototoxicity. H₂ is one of the most plentiful gases in the universe. It is neither explosive nor dangerous at a concentration of less than 4.7% in air and 4.1% in pure oxygen, respectively [10]. Besides hydrogen inhalation, there are other alternative ways to
deliver hydrogen to the cochlea, including hydrogen rich water, hydrogen saturated saline, electrolysed-reduced water, and hydrogen generated from coral calcium hydride solution [29]. H2 has potential for improving the quality of life of patients during chemotherapy by efficiently mitigating the side effects of cisplatin.

Conflict of interest

The authors have declared that no conflict of interest exists.

Acknowledgements

This work was supported by the State Key Program of the National Natural Science Foundation of China (Grant no. 30930098 to Jianhua Qiu), Major State Basic Research Development Program of China (973 Program) (Grant no. 2011CB504505 to Jianhua Qiu), Young Scientists Fund of the National Natural Science Foundation of China (Grants no. 30801287 to Juan Qu; 81101409 to Keliang Xie).

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