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Hydrogen-containing saline attenuates doxorubicin-induced heart failure in rats

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Interactions between doxorubicin (DOX) and iron generate reactive oxygen species and contribute to DOX-induced heart failure. Hydrogen, as a selective antioxidant, is a promising potential therapeutic option for the treatment of a variety of diseases. Therefore, we investigated the preventive effects of hydrogen treatment on DOX-induced heart failure in rats. We found that cardiac function was significantly improved and that the plasma levels of oxidative-stress markers and myocardial autophagic activity were decreased in animals treated with hydrogen-containing saline. Therefore, we conclude that hydrogen-containing saline may have beneficial effects for doxorubicin-induced heart failure.

1. Introduction

Although doxorubicin (DOX) is an effective antineoplastic anthracycline drug, its clinical benefits are limited by significant DOX-induced cardiotoxicity, which can sometimes lead to heart failure. Furthermore, once the heart has been damaged by this drug, few effective treatment options remain available, as doxorubicin-induced cardiomyopathy and heart failure are refractory to conventional therapies (Lefrak et al. 1973). At present, there are no specific treatments for DOX-induced cardiomyopathy. Hydrogen (H_2) has been used as a therapeutic antioxidant in a variety of medical applications, where it acts by selectively reducing cytotoxic oxygen radicals. A large body of work shows that hydrogen possesses not only anti-oxidative properties that can combat stress (Ohsawa et al. 2007) but also a variety of anti-inflammatory (Buchholz et al. 2008) and anti-apoptotic (Chen et al. 2011) properties. Studies in cardiovascular animal models have shown that hydrogen treatment protects against myocardial I/R injury (Hayashida et al. 2008), complications from heart transplantation (Nakao et al. 2010), the development of atherosclerosis (Ohsawa et al. 2008), radiation-induced damage (Qian et al. 2010) and left ventricular hypertrophy (Yu and Zheng 2012). Therefore, we hypothesized that hydrogen-containing saline (HCS) might attenuate doxorubicin-induced heart failure. Here, we demonstrate that hydrogen treatment has cardioprotective effects on DOX-induced heart failure and that it can attenuate DOX-induced autophagy in rats.

2. Investigations and results

Animals were separated into three groups and treated for one month as follows: (1) control group (CON; n = 10); (2) DOX-induced heart failure group (DOX; n = 12); (3) hydrogen-containing saline group (DOX + H_2; n = 11). We investigated the preventive effects of hydrogen treatment on DOX-induced heart failure by observeing effects of H_2 on the survival, echocardiography, histology, the plasma levels of oxidative-stress markers and myocardial autophagic activity of rats.

2.1. Survival rates of rats undergoing HCS therapy

By the end of the HCS therapy, 33.3% of the rats in the DOX group had died, whereas 18.2% of the rats in the DOX + H_2 group had died (Fig. 1A) . No statistically significant differences were observed between the groups with respect to survival rate.

2.2. Effects of H_2 on left ventricular function in a DOX-induced heart-failure animal model

We first studied the effect of H_2 on cardiac function in rats with DOX-induced heart failure. Echocardiography was performed after 4 weeks of HCS treatment (Fig. 1B). LVDD was not significantly different between the groups (CON: 6.27 ± 0.54 mm; DOX: 6.23 ± 0.68 mm; DOX + H_2: 6.25 ± 0.83 mm). However, striking differences were observed between the groups with respect to LVSD (CON: 3.60 ± 0.64 mm; DOX: 4.94 ± 0.58 mm; DOX + H_2: 4.16 ± 0.80 mm), which was significantly increased in the DOX group compared with both the CON and DOX + H_2 groups (CON *vs.* DOX, $P < 0.01$ and DOX *vs.* DOX + H_2, $P < 0.05$). EF (CON: 81.04 ± 6.20 %; DOX: 55.23 ± 4.42 %; DOX + H_2: 66.37 ± 7.19 %) and FS (CON: 45.09 ± 6.58 %; DOX: 25.18 ± 2.34 %; DOX + H_2: 32.38 ± 4.91 %) values were markedly decreased in the DOX group compared with the controls (CON *vs.* DOX, $P < 0.01$ for EF and FS), and HCS treatment improved myocardial performance (DOX vs. DOX + H_2, $P < 0.01$ for EF and FS); however, EF and FS values were lower in the DOX + H_2 group compared with the controls (CON *vs.* DOX + H_2, $P < 0.05$ for EF and FS). Therefore, we conclude that DOX treatment can reduce myocardial performance and that HCS treatment can ameliorate some of these defects in cardiac function.

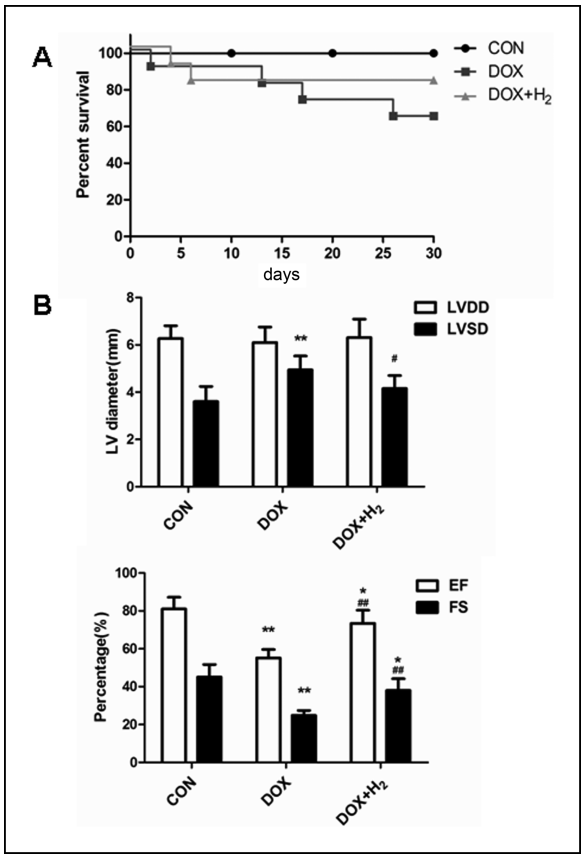

Fig. 1: The effects of H_2 on the survival and echocardiography of rats subjected to DOX-induced heart failure.

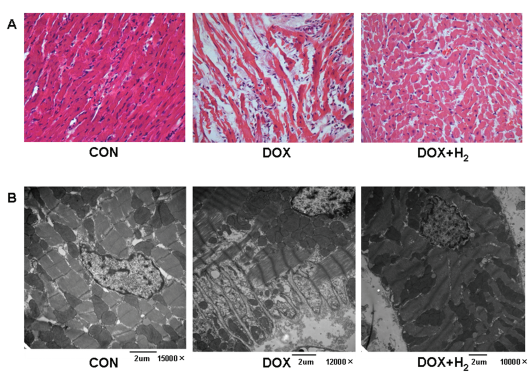

Fig. 2: Representative photomicrographs of H-E staining and electron micrographs of heart tissues.

2.3. *Histology*

Rats in the DOX group showed increased myocardial damage compared with rats in the control group, and this damage was characterized by myocardial rupturing and infiltration of inflammatory cells (Fig. 2A). Rats in the DOX + H_2 group also showed myocardial damage, but not to the same degree as rats in the DOX group (Fig. 2A). Furthermore, we observed myocardial cell lysis and mitochondrial damage in the DOX group (Fig. 2B), whereas the myocardial ultrastructure was more intact following HCS administration (Fig. 2B).

2.4. *Changes in plasma MDA levels*

Plasma MDA levels were measured (CON: 4.64 ± 0.56 mmol/L; DOX: 9.18 ± 1.37 mmol/L;DOX + H_2: 5.05 ± 0.34 mmol/L) (Fig. 3), and we found that MDA concentrations were significantly higher in the DOX group compared with controls

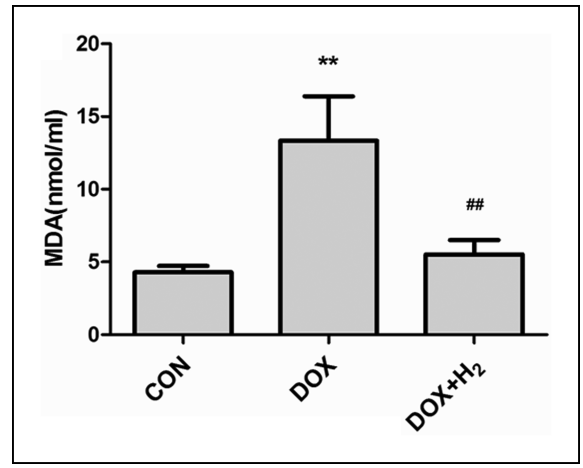

Fig. 3: Plasma MDA levels.

(CON *vs*. DOX, $P < 0.01$). Furthermore, we observed that HCS treatment greatly reduced MDA levels compared with levels in the DOX group (DOX *vs*. DOX + H_2, $P < 0.01$). These findings suggest that H_2 is acting as an antioxidant to decrease the levels of MDA.

2.5. *Effects of H_2 on cardiac autophagy*

We assayed for cardiac autophagy by detecting the induction of LC3B (the LC3-II/LC3-I ratio was used as a marker of autophagy) and Beclin-1 (Fig. 4A and B). We found that LC3-II/LC3-I and Beclin-1 levels were higher in the DOX group compared with the CON and DOX + H_2 groups (CON *vs*. DOX, $P < 0.01$ for LC3-II/LC3-I and Beclin-1; DOX *vs*. DOX + H_2, $P < 0.01$ for LC3-II/LC3-I; DOX *vs*. DOX + H_2, $P < 0.05$ for Beclin-1). In addition, tissue samples from the DOX group showed increased numbers of double-membrane-bound autophagosomes (Fig. 4C), and the average number of autophagosomes found by TEM was positively correlated with LC3-II protein expression in the myocardium.

3. Discussion

In the present study, we investigated the effects of H_2 on oxidative stress and autophagic activity in a rat model of DOX-induced heart-failure, and we came to two main conclusions. First, we could show for the first time that HCS therapy can attenuate doxorubicin-induced heart failure in rats. Second, the effects of H_2 appear to be mediated through autophagic mechanisms.

Mechanisms that have been proposed to explain the cardiotoxic effects of doxorubicin include increased oxidative stress (Richard et al. 2011), changes in myocardial metabolism (Takemura and Fujiwara 2007), interference with molecular signaling pathways (Velez et al. 2011), apoptosis (Nakamura et al. 2000), autophagy (Lu et al. 2009) and inflammation (Liu et al. 2007). In this study, we observed that H_2 treatment led to decreased MDA plasma levels, which suggests that the protective effects of H_2 on DOX-induced heart failure are related to its anti-oxidative properties. Previous studies of the effects of H_2 during oxidative stress-mediated diseases have demonstrated the unique ability of hydrogen to selectively reduce cytotoxic oxygen radicals. However, the beneficial effects of H_2 are unlikely simply due to the removal of oxygen radicals. For example, H_2 can also reduce inflammation (Buchholz et al. 2008) and apoptosis (Chen et al. 2011) in a variety of diseases. Furthermore, H_2 can permeate all regions of the cell, including biomembranes, the cytosol, mitochondria and the nucleus (Ohsawa et al. 2007). Therefore, our results suggest that H_2 inhibits autophagic activity, separately from its causal association with oxidative

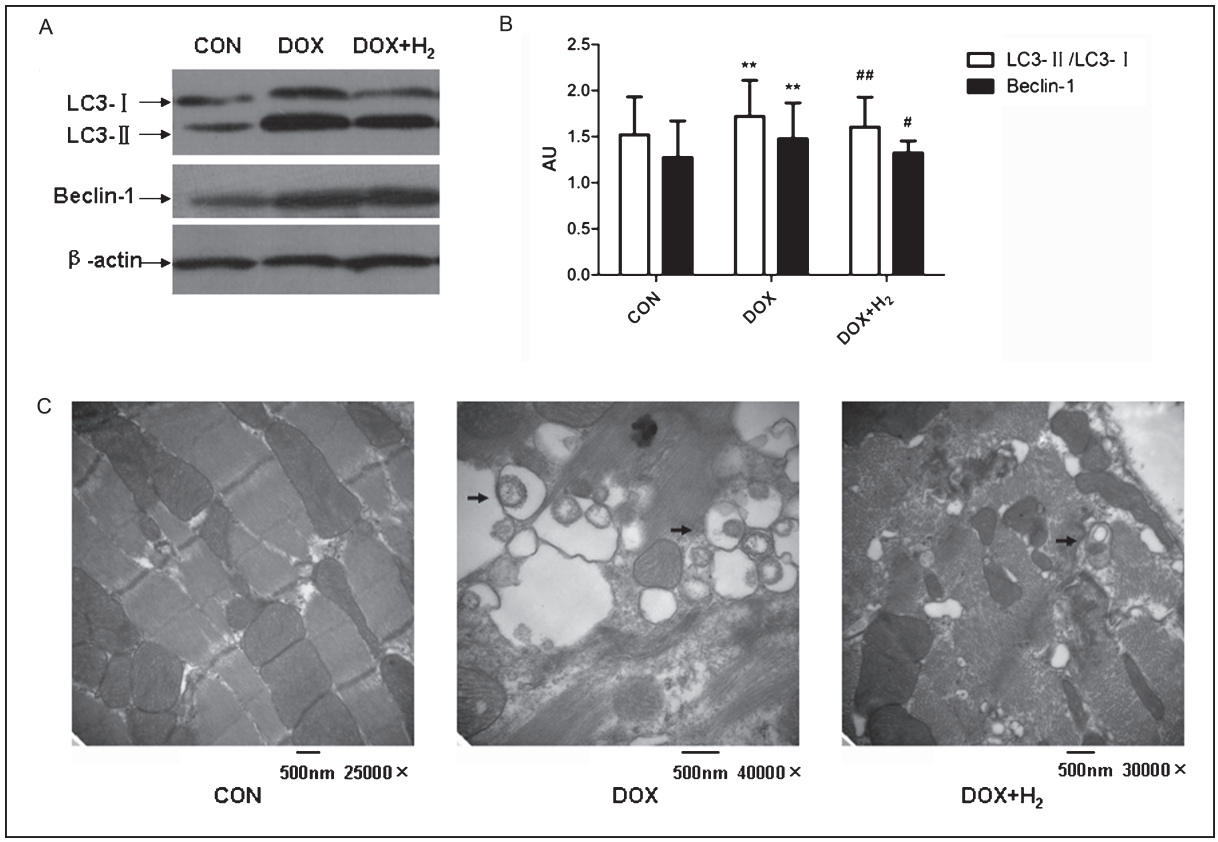

Fig. 4: H_2 inhibits autophagic activity in the heart.

stress, potentially through direct modulation of mitochondrial activity. However, further investigations of the relationship between hydrogen-gas treatment and autophagy in doxorubicin-induced heart failure are required.

Current studies are investigating the effects of doxorubicin in combination with other drugs such as dexrazoxane (ICRF-187) (Lipshultz et al. 2004), ACE inhibitors (Cardinale et al. 2006), β-blockers (Kalay et al. 2006) and antioxidants (Quiles et al. 2002). An ideal antioxidant for use in clinical treatments should be inexpensive, permeable to the cytoplasm and nucleus, and free of toxicity. Along these lines, H_2 possesses significant advantages: (1) unlike most known antioxidants, which are unable to successfully target organelles, H_2 effectively neutralizes cytotoxic oxygen radicals in living cells (James et al. 2005); (2) H_2 is one of the most readily available gases in the natural world, with two common production methods: steam reforming and electrolysis (i.e., water splitting); and (3) as a physiological inert gas, hydrogen has fewer narcotic properties than nitrogen and has no toxicity (Nakao et al. 2009). Considering the ease and safety of administration, hydrogen is a safe and effective antioxidant with minimal side effects that appears to be a good candidate for treating DOX-induced heart failure.

In brief, this study demonstrates that hydrogen-containing saline treatment ameliorates the progression of doxorubicin-induced heart failure in rats, which is likely mediated by its anti-oxidative and anti-autophagic effects. These findings may have far-ranging implications and provide a basis for entirely new types of drug treatment for DOX-induced heart failure and other diseases.

4. Experimental

4.1. Animal protocol

This research was performed in accordance with the National Research Council's protocol for the care and use of laboratory animals. Sixty Wistar rats (12 weeks old), weighing between 200 and 250 g, were used in this study. The animals were maintained under standard laboratory conditions and were provided with free access to rodent chow and water. Ten rats were randomly selected to comprise the control group (CON). The remaining 50 rats were given doxorubicin hydrochloride (DOX) (Meiji Pharmaceutical Co., China) to induce heart failure. Briefly, DOX was delivered via six intraperitoneal injections (each consisting of 2.5 mg/kg DOX dissolved in saline) administered every other day over a period of ~2 weeks (total dose = 15 mg/kg) (Suzuki et al. 2001). After the final injection, the rats were observed for changes in body weight, behavior, general appearance, and mortality over the following 4 weeks.

The animals were divided into three groups and treated for one month as follows: (1) control group (CON; n = 10) – rats were intraperitoneally injected with normal saline (10 ml/kg) daily each morning following the final mock (no DOX) treatment; (2) DOX-induced heart failure group (DOX; n = 12) – rats were intraperitoneally injected with normal saline (10 ml/kg) daily each morning following the final DOX treatment; and (3) hydrogen-containing saline group (DOX + H_2; n = 11) – rats were intraperitoneally injected with hydrogen-rich saline (10 ml/kg) daily each morning following the final DOX treatment.

4.2. Hydrogen-containing saline (HCS) production

HCS was prepared by bubbling H_2 gas (flow rate = 1 L/min) through 500 ml 0.9% saline solution with stirring for 10 min, until the hydrogen reached saturation (Oharazawa et al. 2010). HCS was prepared weekly and stored under atmospheric pressure at 4 °C in an aluminum bag with no empty volume. Gas chromatography was performed to measure the concentrations of hydrogen in the saline and blood using the method described by Ohsawa et al. (2007).

4.3. Echocardiography

Echocardiography was performed after the final DOX treatment and after the completion of the HCS therapy. The rats were anesthetized (0.3–0.5 ml 10% chloral hydrate/100 g, injected intraperitoneally), and the readings were performed by an experienced echocardiographer who was blinded to the group assignments of the rats. Two-dimensional and M-mode images were recorded using a 12-MHz transducer connected to a commercially available echocardiographic system (SONOS 7500, Philips). Images used for measurements were obtained from both the parasternal long- and short-axis views. Left ventricular end-diastolic diameter (LVDD) and left ventricular

end-systolic diameter (LVSD) were measured. Fractional shortening (FS) and eject fraction (EF) were calculated in real time. All measurements are averages from three consecutive cardiac cycles.

4.4. Histopathological observations

Myocardial samples were sliced transversely at the level of the midpapillary muscle into 5-μm-thick sections, which were then stained with hematoxylin-eosin. The samples were assessed by a pathologist in a blinded fashion and scored for the following: myodegeneration, cardiomyocyte hydropic changes, neutrophilic infiltration, hemorrhage, lymphohistiocytic infiltration, and acute myocardial necrosis.

4.5. Transmission electron microscopy (TEM)

Small left-ventricular heart-tissue samples (~1 mm^3) were fixed by immersion in 2.5% glutaraldehyde and processed for TEM according to standard procedures (Popescu et al. 2006).

4.6. Measurement of plasma malondialdehyde (MDA) levels

MDA concentration is a presumptive marker of oxidant-mediated lipid peroxidation. Furthermore, MDA has a long half-life in plasma, and therefore, MDA levels should be effectively cumulative. Plasma MDA concentration was measured using a commercial kit (Jianchen Biological Institute, China).

4.7. Western blotting analysis

Protein concentrations were determined using Bradford assays with bovine serum albumin as a standard. Equal amounts of protein were separated on SDS-polyacrylamide gels, transferred using electro-blotting onto topolyvinylidine difluoride membranes, and incubated with primary antibodies against either LC3B (1:500; CST) or Beclin-1 (1:250; Santa Cruz) overnight at 4 °C with slow rocking. Next, the membranes were incubated with an HRP-conjugated secondary antibody (1:4000) and an HRP-conjugated monoclonal antibody against β-actin (1:4000) for 2 h at room temperature. Immunoreactive bands were detected using chemiluminescence (ECL kit, Amersham Pharmacia). The results are shown as the ratio of the density of the specific bands to the density of the corresponding β-actin band.

4.8. Statistical analysis

Differences in survival between the groups were compared using the Kaplan-Meier survival analysis. All assays were performed using at least three separate experiments in triplicate. Quantitative data are expressed as the mean ± SD values. Statistical analyses of the data were performed using one-way ANOVA, and statistical significance was set at $P < 0.05$.

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