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Oral intake of hydrogen-rich water ameliorated chlorpyrifos-induced neurotoxicity in rats☆

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ABSTRACT

Chronic exposure to low-levels of organophosphate (OP) compounds, such as chlorpyrifos (CPF), induces oxidative stress and could be related to neurological disorders. Hydrogen has been identified as a novel antioxidant which could selectively scavenge hydroxyl radicals. We explore whether intake of hydrogen-rich water (HRW) can protect Wistar rats from CPF-induced neurotoxicity. Rats were gavaged daily with 6.75 mg/kg body weight (1/20 LD_{50}) of CPF and given HRW by oral intake. Nissl staining and electron microscopy results indicated that HRW intake had protective effects on the CPF-induced damage of hippocampal neurons and neuronal mitochondria. Immunostaining results showed that the increased glial fibrillary acidic protein (GFAP) expression in astrocytes induced by CPF exposure can be ameliorated by HRW intake. Moreover, HRW intake also attenuated CPF-induced oxidative stress as evidenced by enhanced level of MDA, accompanied by an increase in GSH level and SOD and CAT activity. Acetylcholinesterase (AChE) activity tests showed significant decrease in brain AChE activity after CPF exposure, and this effect can be ameliorated by HRW intake. An *in vitro* study demonstrated that AChE activity was more intense in HRW than in normal water with or without chlorpyrifos-oxon (CPO), the metabolically-activated form of CPF. These observations suggest that HRW intake can protect rats from CPF-induced neurotoxicity, and the protective effects of hydrogen may be mediated by regulating the oxidant and antioxidant status of rats. Furthermore, this work defines a novel mechanism of biological activity of hydrogen by directly increasing the AChE activity.

Introduction

Organophosphate (OP) compounds are a group of insecticides widely used for the control of agricultural, industrial, home and public health pests (Aardema et al., 2008). Chlorpyrifos (CPF) is one of the most widely used OPs in agricultural settings, despite the restriction of some of its domestic applications by the United States Environmental Protection Agency in 2000 based on human health risk. An epidemiological study of 1000 individuals indicated that up to 82% of U.S. adults had detectable levels of the CPF metabolite (3,5,6,-trichloro-pyridinol) present in their urine (Hill et al., 1995). Although acute high-level exposure to OPs tends to cause severe "cholinergic syndrome" (Costa, 2006), repeated low-level exposure can also produce chronic neurological symptoms and deficits in neurobehavioral performance (Alavanja et al., 2004). Specifically, CPF has been shown to cause mild sensory neuropathy and some memory problems in adults (Kaplan et al., 1993). A recent study compared the neurological symptom of 57 CPF applicators for the cotton crop and 38 non-applicators. CPF applicators had impaired performance compared to controls on the majority of tests on the neurobehavioral battery developed for the study (Khan et al., 2014). Behavioral studies in rodents have identified hippocampus-dependent learning and memory as a target for the neurotoxic effects of repeated subthreshold CPF exposure (Prendergast et al., 1998; Terry et al., 2003).

In the human body, CPF is metabolically activated by oxidative desulfuration to CPF-oxon (CPO), a potent inhibitor of AChE. CPO and related OPs irreversibly inhibit AChE activity by phosphorylation of its active serine site, leading to accumulation of acetylcholine and overstimulation at cholinergic synapses. Other putative mechanisms have also been reported to play an important role in CPF toxicity. Among the additional mechanisms, the induction of oxidative stress has received tremendous focus and attention (Ogut et al., 2011; Saulsbury et al., 2009). Exposure to CPF modifies the endogenous antioxidant defense enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which leads to the development

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of oxidative stress (Bebe and Panemangalore, 2003; Chiapella et al., 2013; Geter et al., 2008).

A recent study has revealed that molecular hydrogen acts as a novel antioxidant which could selectively reduce •OH and $ONOO^-$ but not affect physiological reactive oxygen species (ROS) (Ohsawa et al., 2007). Subsequent studies have have confirmed that consumption of hydrogen reduces oxidative stress in a diverse range of disorders and organ systems including the nervous (Fu et al., 2009; Gu et al., 2010; Li et al., 2010), digestive (Chen et al., 2011; Zheng et al., 2009), cardiovascular (Hayashida et al., 2008; Nakao et al., 2010a) and respiratory systems (Mao et al., 2009). These studies strongly suggest the potential of molecular hydrogen as an effective therapeutic and preventive antioxidant. Drinking hydrogen rich water (HRW) has been considered a safe and convenient mode of delivery for molecular hydrogen. One of the advantages of HRW is its ability to cross the blood–brain barrier and therefore has potential to treat neurological diseases (Li et al., 2010).

In the present study, we hypothesized that oral intake of HRW might play a protective role in the CPF-induced neurotoxicity. We tested our hypothesis using an established adult rat model with oral administration of HRW after chronic exposure to subthreshold dose of CPF. The mechanisms underlying the protective effects of hydrogen have also been investigated.

Materials and methods

Animals. Eight week old male Wistar rats weighing 120–150 g were maintained under standard conditions at 22 °C to 25 °C with a 12 h light-dark cycle and were fed a normal diet. All procedures were conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (China). Before initiating the experiments, the animals were adapted to laboratory conditions. Rats were killed at 4 time points: 2, 4, 6, and 8 weeks. CPF (Sigma, St. Louis, MO, 99.2% purity) was prepared by corn oil (2 ml/kg) to obtain an effective concentration of 6.75 mg/kg body weight (1/20 LD_{50}). CPF or corn oil was administered once daily by oral gavage for a period of 2–8 weeks. HRW or normal water was changed every morning and night and rats drank freely during the dosing period. At each time point, rats were randomly divided into three groups. Group I (C/oil): Rats in the control group gavaged with corn oil only and given normal water. Group II (CPF): Rats gavaged with CPF and given normal water. Group III (HRW): Rats gavaged with CPF and given HRW.

HRW preparation. Hydrogen-rich water (termed HRW) was prepared as described previously (Nakao et al., 2010b). Briefly, a plastic shelled product (termed hydrogen water stick) consisting of metallic magnesium (99.9% pure) and natural stones in polypropylene containers combined with ceramics (Doctor SUISOSUI®; Friendear Inc., Tokyo, Japan) was used to produce hydrogen. The hydrogen water sticks were placed into distilled water for more than 6 h before use at room temperature. The hydrogen concentration was monitored by using a needle-type Hydrogen Sensor (Unisense A/S, Aarhus, Denmark) every week. The hydrogen water sticks were replaced every two weeks to ensure maintenance of hydrogen concentration (more than 600 μM).

In an *in vitro* study, hydrogen-rich water provided by Beijing Hydrovita Beverage Co., Ltd. (termed H-HRW) was used to determine the effect of hydrogen on AChE activity.

A ContrAA 700 high resolution continuum source atomic absorption spectrophotometer (Analytik Jena, Jena, Germany) was used for magnesium measurement. The instrument settings were: absorption line 285.2 nm and width 0.2 nm. The pH value was measured with an Orion model 420A pH meter (Boston, MA, USA).

Tissue preparation. At each time point, rats were anesthetized with isofluorane, the brain tissue was carefully excised and washed with ice-cold physiologic saline (0.9%, w/v). One-half of the brain was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and embedded in paraffin for Nissl staining and immunochemistry. The hippocampus dissected from the other half of the brain was stored at −80 °C until use for oxidative stress biomarkers and AChE activity analysis. Hippocampal tissues (30 mg) were homogenized in 0.3 ml RIPA buffer (Beijing Puli Lai Gene Technology Co., Ltd., Beijing, China) and then centrifuged at 8000 *g* for 10 min, the supernatant was used for the determination of oxidative stress biomarkers and AChE activity. For electron microscopy, rats were sacrificed *via* transaortic perfusion of 2.5% glutaraldehyde and their brains were removed. The hippocampus was dissected and cut into 1 mm × 2 mm × 1 mm blocks before being kept in the same fixative overnight.

Nissl staining and immunochemistry. Serial sections (8 μm) were cut coronally through the cerebrum containing the hippocampus. For Nissl staining, every subsequent 10th section (3 sections in each animal) was collected. Toluidine blue was used to stain the Nissl body in the neurons. Mounted slides were examined and photographed under an Olympus light microscope. Six random visual fields of the hippocampal cornu ammonis 1 (CA1), cornu ammonis 1 (CA3) and dentate gyrus (DG) were photographed in each section. The number of staining cells in each field was counted at high magnification (×400). The data were represented as the number of cells per high-power field. Alternatively, every subsequent 12th section (3 sections in each animal) was collected for GFAP staining. Sections were treated with 3% hydrogen peroxide for 10 min to inactivate endogenous peroxidases. The sections were incubated with 10% normal goat serum. After the blocking serum was removed, sections were immunostained overnight at 4 °C using a mouse monoclonal antibody against GFAP (Beyotime, China) to assess astrocyte activation, then with biotinylated secondary antibody at 37 °C for 20 min. The GFAP-positive cells in the hippocampus were detected using streptavidin–biotin complex (SABC) and DAB kits (Zhongshan, China). Six random high magnification fields (×400) per section were counted to evaluate for GFAP-positive cells. The GFAP-positive cell count per mm^2 tissue area was calculated using Image-Pro Plus software.

Electron microscopy. Hippocampal regions of interest (CA1, CA3 and DG) were identified through light microscopy. The ultra thin sections were cut at 0.1 μm and stained with uranyl acetate and lead citrate. A Hitachi H-7650 transmission electron microscope was used to capture images. Mitochondria per cell in the same hippocampal region in different treatment groups were quantified using grid counting by three blinded raters.

Measurement of oxidative stress biomarkers. Some biomarkers were determined to evaluate the oxidative stress status in brain. (1) Malondialdehyde (MDA) levels were measured using a thiobarbituric acid reactive species (TBARS) assay kit (Cayman, USA). The total protein concentration was determined by the Bradford Assay (Tiangen, China) using BSA as a standard. Results are expressed as [MDA] in nmol/mg of protein. (2) Glutathione (GSH) was determined according to the recycling system by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and GSH (Dojindo, Japan). Concentration of GSH in sample solutions was determined using a calibration curve, and expressed as nmol/mg protein. (3) SOD activity was measured with assay kits (Dojindo, Japan), according to the manufacturer's protocol. (4) CAT activity was assayed according to the method of Goth and expressed as mU/mg protein by the rate of decrease of hydrogen peroxide (Goth, 1991).

Determination of the AChE activity in vivo. The supernatant of the brain tissue homogenate was diluted with 0.1 M phosphate buffered saline (PBS, pH 7.4). The enzyme activity assay was carried out in a