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Hepatorenal protective effects of medicinal herbs in An-Gong-Niu-Huang Wan (AGNH) against cinnabar- and realgar-induced oxidative stress and inflammatory damage in mice

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ABSTRACT

An-Gong-Niu-Huang Wan (AGNH) is a famous traditional Chinese medicine prescription that contains cinnabar (HgS) and realgar (As_2S_2); the clinical practice of AGNH is hindered because both mercury and arsenic are hepatorenal toxic metalloids. It is noted that the cinnabar and realgar in AGNH are not used alone, but rather combined with different kinds of medicinal herbs as a formula to use. In this study, we evaluated the hepatorenal protective effects of the medicinal herbs in AGNH after co-exposure to cinnabar and realgar for 4 weeks in mice. The combination of the herbs in AGNH alleviated cinnabar and realgar-induced histopathological alterations and oxidative stress in the liver and kidneys. Furthermore, in cinnabar and realgar-treated mice, the increased expression levels of inducible enzymes (COX-2 and iNOS) and proinflammatory mediators (IL-1β, TNF-α, PGE2 and NO) in the liver and kidneys were consistently down-regulated when medicinal herbs were combined as a formula. We also found that the herbs could reduce the inflammatory response by the inactivation of the MAPK and PI3K/Akt signaling pathway and the resulting blockade of NF-κB activation. Overall, our data indicates that the herbal medicines in AGNH attenuate cinnabar and realgar-induced hepatorenal toxicity by improving antioxidant competence and suppressing inflammatory injury.

1. Introduction

An-Gong-Niu-Huang Wan (AGNH) is a composite prescription of traditional Chinese medicine (TCM) composed of *Calculus Bovis*, *Cornu Bubali*, *Moschus*, *Margarita*, *Cinnabaris*, *Realgar*, *Rhizoma Coptidis*, *Radix Scutellariae Baicalensis*, *Fructus Gradeniae*, *Radix Curcumae* and *Borneolum Synthcticum* (Zhao et al., 2015). In China, AGNH is generally prescribed for patients suffering from acute and chronic cerebral diseases, such as hypoxic-ischemic encephalopathy, viral encephalitis, cerebral paralysis, hypertensive cerebral hemorrhage, severe craniocerebral trauma and diffuse axonal injury (Ding and He, 1986; Wang et al., 2014). However, the clinical application of AGNH is limited by the existence of two known hepatorenal toxic metalloids, mercury (Hg) and arsenic (As) found in cinnabar (contains 96% HgS) and realgar (contains 90% As_2S_2), respectively (Wang et al., 2013, 2015a; Wei et al., 2009).

In recent years, it was reported that cinnabar and realgar, as well as AGNH, protected dopaminergic neurons against lipopolysaccharide-induced neurotoxicity by inhibiting the microglia activation and proinflammatory factor production (Zhang et al., 2012a). Moreover, cinnabar and realgar exerted similar effects as AGNH on changes in levels of cortical catecholamine and its metabolites in endotoxin-induced intracerebral hemorrhage in rats (Zhu et al., 2007). Evidence from *in vitro* and *in vivo* studies suggest that both cinnabar and realgar are probably the main contributors to the neuroprotective effect of AGNH.

Because cinnabar and realgar are relatively poorly water-soluble in the form of sulfides and have long history of safe use at therapeutic doses, they are considered to be less poisonous than common mercurials (mercuric chloride and methylmercury) and arsenicals (arsenite and arsenate) (Liu et al., 2008a, 2008b; Wu et al., 2011). In addition, both the cinnabar and realgar almost cannot cross this blood-brain barrier because they cannot pass through slit junctions and have

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difficulty traversing the lipid cell membrane. However, in the liver and kindey tissues, a large part of the basement membrane is exposed due to large discontinuous capillaries, through which most small molecule drugs can pass into the hepatic and renal interstitium. Thus, the liver and kidneys have been suggested as the main target organs of cinnabar and realgar-induced toxicity. Long-term use or overdose of cinnabar or cinnabar-containing preparations could induce hepatic and renal dysfunction due to the accumulation of mercury being present as non-sulfur bound species (Huang et al., 2005). When animals were exposed continuously and chronically to cinnabar at clinical dosage, free mercury could be released and absorbed, causing a pronounced depletion of sulfur groups of enzymes, oxidizing intramitochondrial NADH and NADPH, and enhancing depolarization of the mitochondrial inner membrane, hence leading to reactive oxygen species (ROS) generation and lipid peroxidation (LPO) (Bridges et al., 2012; Lin et al., 1996). On the other hand, soluble arsenic contents of realgar were measured (0.6% of the total arsenic content) under simulated gastric fluid (Kwan et al., 2001). If realgar-containing medicines were taken for long periods of time or in excessive dosages, soluble forms of arsenic could also be absorbed from the gastrointestinal tract into blood circulation, freely crossing cell membrane and finding distribution in target organs, especially the liver and kidneys (García-Sevillano et al., 2014). Similar to mercury, the soluble arsenic in realgar could accelerate production of oxygen-derived free radicals (such as H_2O_2 and superoxide) by binding to biological ligands containing sulfhydryl groups especially vicinal thiols, leading to oxidation of DNA and proteins and oxidative damage to the liver and kidneys (Flora, 2011; Yadav et al., 2012). Hence, oxidative stress plays a seminal role in the pathogenesis of chronic liver and kidney injury elicited by mercury-containing cinnabar and arsenic-containing realgar.

Chronic inflammation is also an important pathological mechanism underlying the progression of mercury and arsenic-induced hepatic and renal lesions (Peters et al., 2015; Wang et al., 2015c; Yang et al., 2016). Studies in the past decades have demonstrated that mercury and arsenic generate ROS that trigger activation of pro-inflammatory signals, including mitogen-activated protein kinase superfamily members (MAPK), phosphatidylinositol 3 kinase (PI3K), Akt and nuclear factor-kappa B (NF-κB) (Chen et al., 2016a; Gong et al., 2015; Yang et al., 2016). It has been reported that mercury and arsenic-activated MAPK/NF-κB and PI3K/Akt/NF-κB signal pathways play critical roles in the multiple steps of inflammatory responses by regulating the transcription of a chain of pro-inflammatory mediators (Chen et al., 2016a; Duan et al., 2017; Sun et al., 2017). Considering the pro-oxidative and pro-inflammatory properties of cinnabar and realgar, antioxidant and/or anti-inflammatory intervention might represent an effective strategy to ameliorate cinnabar and realgar-induced hepatorenal toxicity.

As noted previously, cinnabar and realgar are not used alone in AGNH, rather they are combined with other herbs as adjuvants in the form of a composite formula. Previously published studies described that multiple constituents of four combined herbs in AGNH, including *Radix Scutellariae Baicalensis* flavones, *Rhizoma Coptidis* alkaloids, *Fructus Gradeniae* iridoids and *Radix Curcumae* curcuminoids, contributed to protective effects against both liver and kidney injury owing to their anti-oxidative and anti-inflammatory properties (Adil et al., 2016; García-Niño and Pedraza-Chaverrí, 2014; Liao et al., 2017; Rong et al., 2017). However, the ameliorative effects of the above mentioned herbs in AGNH to cinnabar and realgar-induced hepatorenal toxicity were still not investigated. The aim of the present study was to investigate the potential detoxification mechanism by which the medicinal herbs in AGNH elicited their hepatorenal protective effects in mice challenged with cinnabar and realgar-intoxication, mainly focusing on histopathological alterations, oxidative stress and signaling pathways involved in pro-inflammatory mediator and cytokine expression.

2. Materials and methods

2.1. Materials

An-Gong-Niu-Huang Wan (AGNH, 3 g per pill), cinnabar and realgar were provided from Guangzhou Bai-Yun-Shan Zhong-Yi Pharmaceutical Company Ltd. (Guangzhou, China). Primary antibodies against the following antigens were as follows: CD11b and F4/80 (BioLegend, San Diego, CA, USA); CD67 (Abcam, Cambridge, MA, UK); cyclooxygenase 2 (COX-2; Boster biological technology, Wuhan, China); phospho-(Thr183/Tyr185)-stress-activated protein kinase (SAPK)/c-Jun NH2 kinase (JNK), phospho-(Thr202/Tyr204)-ERK1/2, phospho-(Thr180/Tyr182)-p38 MAPK, JNK, ERK1/2 and Akt (Cell Signaling Technology, Beverly, MA, USA); phospho-(Thr308)-Akt, inducible nitric oxide synthase (iNOS) and lamin B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), phospho-(Tyr467)-PI3K p85/phospho-(Tyr199)-p55, PI3K, p38 MAPK, IκB kinase α/β (IKKα/β), phospho-(Ser180/181)-IKKα/β, NF-κB p65, phospho-(Ser32/Ser36)-IκBα, IκBα and β-actin (Bioworld Technology, Minneapolis, MN, USA). Malondialdehyde (MDA), catalase (CAT) and nitric oxide (NO) assay kits were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). H_2O_2, reduced glutathione (GSH) and myeloperoxidase (MPO) assay kits were provided from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated.

2.2. Animals

Male and female Kunming mice, weighing 20 ± 2 g, were purchased from the Experimental Animal Center of Third Military Medical University (Chongqing, China). All mice were housed under standard conditions of humidity (45 ± 5%), temperature (24 ± 1 °C) and a 12-h alternate light/dark cycle. They were fed standard rodent chow diet and drinking water *ad libitum*, and acclimatized for 1 week prior to use. The animal care procedures were approved by the Institutional Ethics Committee of the Chongqing University of Technology. All experiments were conducted in full compliance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals (8th Edition, 2011).

2.3. Experimental protocol

A total of 36 mice were randomly divided into 3 groups of 12 mice. Animals of the first group received saline (vehicle), while those of the second group received AGNH (2.5 g/kg) by oral administration once daily for 28 consecutive days. AGNH at 2.5 g/kg is equivalent to a 5-fold higher clinical dosage. The current dose of AGNH was set on the basis of the results from our pilot experiments. According to Pharmacopoeia of China (2015 edition), per gram of AGNH contains equal amounts (0.056 g) of cinnabar and realgar. For comparison, the third group of animals was orally administrated cinnabar (0.14 g/kg) and realgar (0.14 g/kg) daily. On day 28, mice were sacrificed under pentobarbital sodium (50 mg/kg) anesthesia at 1 h following the last dosing. Blood was collected by eye bleeding, left 1 h to clot, and centrifuged at 1500 g at 4 °C for 10 min for serum separation. The isolated serum was stored at −80 °C for measuring cytokine (IL-1β and TNF-α) levels. For protein analysis, a portion of liver and kidney specimens were dissected, snap-frozen in liquid nitrogen, and stored at −80 °C. The left lateral lobe of the liver and left kidney was fixed in 4% paraformaldehyde and embedded in paraffin for 4 μm sections. The remaining liver and kidney tissues were weighed and homogenized to obtain 10% (*w/v*) homogenate in ice-cold physiological saline or lysis buffer containing 150 mM NaCl, 20 mM Tris pH 7.5, 1% (*w/v*) Triton X-100, 5 mM EDTA, 1 M DTT, 100 mM PMSF, 10 mg/ml leupeptin and aprotinin (Beyotime). The homogenates were centrifuged at 14000 g for