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Combination therapy with andrographolide and D-penicillamine enhanced therapeutic advantage over monotherapy with D-penicillamine in attenuating fibrogenic response and cell death in the periportal zone of liver in rats during copper toxicosis

Dijendra Nath Roy, Gargi Sen, Kaustav Dutta Chowdhury, Tuli Biswas *

Cell Biology and Physiology Division, Indian Institute of Chemical Biology, A Unit of Council of Scientific and Industrial Research, 4, Raja S.C. Mullick Road, Kolkata 700032, India

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ABSTRACT

Long treatment regime with D-penicillamine is needed before it can exert clinically meaningful benefits in the treatment of copper toxicosis. The consequence of long-term D-penicillamine treatment is associated with numerous side effects. The limitations of D-penicillamine monotherapy prompted us to search for more effective treatment strategies that could decrease the duration of D-penicillamine therapy. The present study was designed to evaluate the therapeutic potential of D-penicillamine in combination with another hepatoprotective drug, andrographolide in treatment of copper toxicosis in rats. D-penicillamine treatment led to the excretion of copper through urine. Addition of andrographolide to D-penicillamine regime appeared to increase protection of liver by increasing the biliary excretion of copper and reduction in cholestatic injury. The early removal of the causative agent copper during combination treatment was the most effective therapeutic intervention that contributed to the early rectification of fibrosis in liver. Combination treatment reduced Kupffer cells accumulation and TNFα production in liver of copper exposed rats. In particular, andrographolide mediated the anti-inflammatory effect by inhibiting the cytokine production. However, another possible mechanism of cytoprotection of andrographolide was decreasing mitochondrial production of superoxide anions that resulted in better restoration of mitochondrial dysfunction during combination therapy than monotherapy. Furthermore, ROS inhibition by combination regimen resulted in significant decline in activation of caspase cascade. Inhibition of caspases attenuated apoptosis of hepatocytes, induced by chronic copper exposure. In summary, this study suggested that added benefit of combination treatment over use of either agent alone in alleviating the hepatotoxicity and fibrosis associated with copper toxicosis.

Introduction

The liver is a major target organ in copper toxicity, where it tends to build up, causing diseases like Indian childhood cirrhosis, idiopathic copper toxicosis and tyrolean infantile cirrhosis (Tanner, 1998; Müller et al., 1996; Muller et al., 1998). The increased incidence of chronic copper toxicosis in young children as compared to adults reflects the inefficient mechanisms of the copper excretion from liver in children due to impaired biliary excretion leading to copper accumulation in liver (Scheinberg and Sternlieb, 1994). Distortion of small biliary branches resulting in disturbances of bile flow leads to cholestasis which causes deposition of copper granules in parenchymal cell cytoplasm (Elmes et al., 1989; Kaplan, 1996). Zonal heterogeneity in copper accumulation exists within the hepatic acinus showing pericanalicular deposition of copper is more in the periportal (PP) zone than those in the pericentral area (Fuentealba et al., 1989; Roy et al., 2009). Apart from cytoplasm, copper accumulates in several cell compartments of hepatocytes and has a distinct effect on mitochondria morphology and function (Lindquist, 1968; Roy et al., 2009). Accumulation of copper in mitochondria is a consequence of the special requirements of the organelle to utilize copper for the synthesis of enzymes such as copper cytochrome oxidase, and to regulate copper homeostasis at the optimum level through biliary excretion (Leary et al., 2009; Prohaska, 2008). However, loss of homeostasis resulting in accumulation of copper in the mitochondria has significant toxic consequences, both for the mitochondrion itself as well as hepatocytes as a whole (Roy et al., 2009). Hepatocytes located in PP zone undergo apoptosis and gradually degenerative

Abbreviations: ALT, alanine transaminase; AG, Andrographoliode; D-Pen, D-penicillamine; DMSO, dimethyl sulphoxide; DTT, dithiothreitol; DCFH-DA, 2′,7′-Dichlorodihydrofluorescein diacetate; GGT, Gamma-glutamyl transpeptidase; SDS, sodium dodecyl sulphate; TNFR1, tumour necrosis factor receptor 1; TNFα, tumour necrosis factor-alpha; ROS, Reactive oxygen species; PP, Periportal.

* Corresponding author. Cell Biology and Physiology Division, Indian Institute of Chemical Biology, CSIR, 4, Raja S.C. Mullick Road, Jadavpur, Kolkata, Pin-700032, India. Fax: +91 33 2473 0284.
E-mail address: tulibiswas@iicb.res.in (T. Biswas).

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lesions become evident in this area, which progresses to widespread inflammation of liver with focal necrosis (Masuda et al., 1988). Hepatic collagen infiltration, along with pathological features similar to liver cirrhosis is observed at later stages of copper toxicosis (Thornburg et al., 1986; Herrtage et al., 1987; Toyokuni et al., 1989).

The intracellular nature of signals that might regulate hepatic conditioning in setting of chronic copper exposure, are likely to be highly complex but the common theme that has now emerged is the role of reactive oxygen species (ROS) in the pathogenesis of copper-induced hepatic fibrosis (Muriel, 2009; Das Sarma et al., 2010). The critical event in hepatic fibrosis is activation of stellate cells, which is mediated by the lipid peroxides formation in the membrane and endoplasmic reticulum of the hepatocyte after getting injured by ROS (George et al., 2003). These activated stellate cells are the main source of extracellular matrix formation in liver tissue leading to liver fibrosis (Rojkind et al., 1979; Jarnagin et al., 1994). Copper increases Kupffer cell-dependent oxygen utilization by promoting the free radical processes related to the respiratory burst of activated liver macrophages, which contribute to the concomitant development of hepatocellular injury. The activation of inflammatory cytokines such as tumour necrosis alpha (TNFα), inter leukin-6, inter leukin-8 through Kupffer cell-mediated responses during copper accumulation in liver contributes to pathogenesis of steatosis and fibrosing steatohepatitis (Videla et al., 2003; Kohgo et al., 2007).

A complementary approach that delineates the treatment of copper-induced hepatotoxicity includes the use of chelators, superoxide dismutase (SOD) and catalase mimics, antioxidants and cytokine antagonists (Fuentealba and Aburto, 2003). D-penicillamine (D-Pen) and trientine, slow-acting chelators are presently the first choice in the treatment of copper toxicosis (Fuentealba and Aburto, 2003). Prophylactic enterally administered D-Pen is well tolerated and does not have any major adverse effects during short-term use. But, the negative effects appear after the long-term use (Scheinberg, 1968). Collagen and elastin crosslinking are inhibited during long term use of D-Pen treatment, which results in thin and vulnerable skin, cutis laxa, elastosis perforans serpiginosa (Grasedyck, 1988). Long term toxic influences include pancytopenia, gastrointestinal disturbances, changes or loss of taste and proteinuria (Grasedyck, 1988). Treatment with either SOD or catalase mimetic can only suppress a little part of toxic oxygen species that is generated by the induction of copper. Despite clinical benefit, these enzymes suffer as drug candidates due to several reasons because of immunogenic responses. Nonenzymatic supplementation with N-acetyl cysteine as protective strategy in copper toxicosis has also resulted in conflicting outcomes in experimental models (Patterson et al., 2003). Administration of antioxidants such as α-tocopherol provided minimal protection against ROS in liver diseases (Tucker ad Townsend, 2005). Less than half of patients undergoing treatment with TNF antagonists alone achieved complete remission in liver diseases (Domm et al., 2008). Therefore, the present challenge is to counter copper induced oxidative attack and to identify a nontoxic cytokine inhibitor that can target the liver in combination manner with D-Pen, so that the therapy regime of drug against copper toxicosis is shortened.

Andrographolide (AG) is a potential therapeutic diterpenoid lactone exerting choloretic and anti-cholestatic effects (Tripathi and Tripathi, 1991). It is an alternative drug widely used as a single agent or combined with other drugs for better therapeutic efficacy in several diseases (Jarukamjorn and Nemoto, 2008). AG can scavenge superoxide molecules and thwart premature apoptosis of cells (Shen et al., 2002). Bile acid-induced cellular apoptosis has an important role in the pathogenesis of cholestatic liver disease (Becker et al., 2007). AG is known to decrease the intrahepatic protein levels associated with fibrogenesis during liver diseases (Kwo et al., 1995). AG decreased the phorbol-12-myristate-13-acetate plus calcium ionophore A23187, stimulated proinflammatory cytokine gene expression and production of TNFα (Qin et al., 2006). Our recent studies have demonstrated that an analog of AG (14-deoxyandropapholide) desensitized hepatocytes to TNFα induced apoptosis through the release of its receptor (TNFR1) from hepatocytes (Roy et al., 2010).

Based on the mechanisms of action of two drugs, D-Pen increases excretion of copper through urine and AG exerts choloretic and anti-cholestatic effects—this hypothesis predicts that simultaneous administration of D-Pen and AG would cause fast removal of copper from liver during copper toxicosis in rats. At the end point of this joint action, they might downregulate copper induced inflammatory and pathological changes in the liver. Thus, we examined in an *in vivo* experimental model of copper toxicosis whether the addition of AG to D-Pen would be beneficial for its hepatoprotective effect and would reduce the time span of treatment. Moreover, the aim of the present study was to investigate, if this combination was capable in reversing the fibrotic changes in liver that occurred during copper toxicosis.

Materials and methods

Reagents. Unless otherwise noted, all chemicals were obtained from Sigma (St. Louis, MO, USA). Antibodies against CD68 (6A324), Bid (D-19) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA. USA). Anti TNFα antibody, Active caspase-3,-8,-9 antibodies, α smooth muscle actin (αSMA) antibody were purchased from Abcam (Abcam Inc., Cambridge, USA). Andrographolide was purchased from Sigma-Aldrich, St. Louis, USA.

Preparation of drugs. AG (100 mg) was dissolved in 0.5 ml DMSO and was diluted with PBS (5% DMSO and 95% PBS, pH 7.4) to achieve the concentration required for *in vivo* studies. D-Pen stock solution was prepared by dissolving 100 mg of D-Pen in 1 ml water. These drugs were prepared freshly on every third day during the period of treatment.

Animals and treatments. Male Sprauge–Dawley rats (45–50 g) were given standard laboratory diet and water *ad libitum*. All animals were bred and housed in the animal house of Indian Institute of Chemical Biology, Kolkata. They were kept in an environment of constant temperature and humidity with 12 h day–night cycle. All animal studies were performed following the mandates approved by Animal Ethics Committee (Committee for the purpose of control and supervisions of experiments of animals, Govt. of India).

All rats were allowed 5 days to acclimate the laboratory conditions and then these animals were divided into eleven groups with 6 animals in each group. Control animals included 5 groups. These groups were administered daily with 0.1 ml of saline, drug vehicle solution (0.1 ml of 5% DMSO and 95% PBS, pH 7.4), D-Pen (100 mg/kg body weight/day), AG (20 mg/kg body weight/day), and combination of both the drugs respectively. Saline was fed for a period of 75 days starting from the initial day of the experiment. Drug vehicle (DMSO) was administered in a similar routine as drug treated groups. The control rats were initially kept unexposed for 45 days and then given drugs orally once daily at 2:00 p.m–3:00 p.m. for the subsequent 15 days. Rats were exposed to copper sulfate solution, (15 mg/kg body weight/day, dissolved in saline) by gavage daily for a period of 45 days and kept unexposed for next 30 days. Vehicle (DMSO) treated copper exposed rats were initially gavaged with copper sulfate (15 mg/kg body weight/day, dissolved in saline) for 45 days and then administered DMSO orally for next 30 days. Animals in the drug treated groups were orally gavaged with respective drugs (D-Pen or/and AG) after being initially exposed to copper (15 mg/kg body weight/day) for 45 days. Copper exposed rats were treated with AG by gavage for 30 consecutive days (at the doses of 5, 10 and 20 mg/kg body weight/day). No sign of toxicity was noticed on the behavior and general health of the animals when exposed to AG. Another group of animals exposed to copper (15 mg/kg body weight/day) for 45 days was administered orally with D-Pen (100 mg/kg body weight/day, dissolved in water) for a period of next 30 days. In combined treatment group,