Effects of arecoline on hepatic cytochrome P450 activity and oxidative stress

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ABSTRACT — Betel-quid use is associated with the risk of liver cirrhosis and hepatocellular carcinoma. The aim of the present work was to evaluate the impact of arecoline on human hepatic cytochrome P450 (CYP) enzymes in vitro and rat hepatic CYP enzymes, as well as the hepatic oxidative stress and liver injury of rats in vivo. The in vitro results indicated that arecoline hydrobromide (AH) has no significant effect on the activities of CYP2B, 2C9, 3A4, 1A2, 2E1 and 2D6 in human liver microsome (HLM). However, oral administration of AH at 4 and 20 mg/kg/d for seven consecutive days significantly increased the activities of rat hepatic CYP2B, 2E1, 2D, 3A, 2C and 1A2. In addition, AH at 100 mg/kg/d significantly increased the levels of ALT, AST and MDA, decreased the levels of SOD, CAT, GSH-Px and GSH, in rat liver. The in vivo induction of AH on rat hepatic CYP isoforms suggested that the high risk of metabolic interaction should be existed when the substrate drugs of the six kinds of CYP isoforms was administered in betel-quid use human. Furthermore, the in vivo results also suggested that AH-induced hepatotoxicity should be associated with the induction of AH on rat hepatic CYP2E1 and 2B.

Key words: Cytochrome P450 enzyme, Arecoline, Oxidative stress

INTRODUCTION

Betel-quid use is associated with oral cancer, obesity, diabetes mellitus, hypertension, hyperlipidemia, liver cirrhosis, hepatocellular carcinoma, and spermatogenic damage (Tsai et al., 2004; Guh et al., 2006; Wu et al., 2010). Arecoline (Fig. 1), the main Areca alkaloid of the betel nut, is reported to have cytotoxic and genotoxic effects in non-liver cells (Kumpawat et al., 2003; Chou et al., 2009), hepatocytes (Kevekordes et al., 2001; Chou et al., 2008) and mice (Dasgupta et al., 2006), as well as hepatotoxicity and testicular toxicity in mice and rats in vivo (Dasgupta et al., 2006; Zhou et al., 2014). However, Cheng et al. reported that arecoline induces the death of HA22T/VGH hepatoma cells, but not normal hepatocytes. This study highlights the possibility that low-dose arecoline might be useful in the treatment of hepatoma and provides clues for studying the arecoline-induced detachment of hepatoma cells (Cheng et al., 2010).

Betel-quid is used by approximately 10% of the world population. Herbal induction or inhibition of one or more drug-metabolizing enzymes can lead to changes in clearance or toxicity of a co-administered drug or of the herb itself (Bjornsson et al., 2003; Zhao et al., 2012). CYP3A4 is one of the most important hepatic metabolic enzymes with broad substrate specificity. CYP2D6 is involved in the metabolism of numerous centrally acting drugs, endogenous neurochemicals and in the inactivation of neurotoxins. CYP2C9 participates in the hydroxylation of many important acidic drugs. CYP1A2 is able to metabolize some polycyclic aromatic hydrocarbons to carcinogenic intermediates. CYP2E1 participates in the metabolic activation process of many poisons and pre-carcinogens. CYP2B is important for the biotransformation of a large number of drugs, endogenous neurochemicals and toxins. Arecoline can induce profound metabolic effects by increasing the hepatic levels of cytochrome b5 and cytochrome P-450 (Singh and Rao, 1993; Singh et al., 2000), but the effect of arecoline on the activities of hepatic CYP enzymes, has not been worked out in detail. Here we report the effects of AH on (a) the activities of rat hepatic CYP2B, 2C, 3A 1A2, 2E1 and 2D in vivo, (b) the activities of human hepatic CYP2B, 2C9, 3A4, 1A2, 2E1 and 2D6 in vitro, (c) hepatic oxidative stress and liver injury in vivo. Our study may help to design proper therapeutic and prevent measures for arecoline-induced hepatotoxicity in the future.
MATERIALS AND METHODS

Chemicals and reagents
Arecoline hydrobromide (AH) was purchased from Aladdin Chemistry Co. Ltd. (Shanghai, China). Bupro- pion was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Testosterone and tolbuta- mide were purchased from Dr. Ehrenstorfer (Ausberg, Germany). 4-hydroxytolbutamide, 6-hydroxychlorzoxa- zone and dextromethorphan were purchased from TRC (Toronto, Canada). 6β-hydroxytestosterone and bupropion were purchased from Cayman Chemical Company. Nicotima- mide adenine dinucleotide phosphate reduced (NADPH) was purchased from Roche Co. (Basel, Switzerland). Dextromethorphan was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Phenacetin was pur- chased from Alfa Aesar (Woburn, MA, USA). 4-aceta- midophenol was purchased from TCI (Tokyo, Japan). BCA Protein Assay Kit was purchased from Thermo Scientific (Rockford, IL, USA). Hydroxybupropion was purchased from Cayman Chemical Company. Pooled HLM was purchased from BD Gentest (Woburn, MA, USA). Ethyl acetate, methanol and acetonitrile were HPLC grade and purchased from Merck (Darmstadt, Germany). The other chemical reagents were of analyti- cal grade or better.

Animal treatments and preparation of liver microsomes
Male Wistar rats (200-250 g) were purchased from the provincial Disease Prevention and Control Center of Hubei and were maintained in SPF animal room at temperature 22 ± 2, 60 ± 5% humidity and 12/12 hr day/night cycle. All procedures were approved by Ethic Committee of Hubei University, and complied with health guidelines for the care and use of laboratory animals. Rats were fasted for at least 12 hr prior to initiating the experiments and had free access to water.

A total of 24 rats were randomly divided into 4 groups with 6 rats in each group to receive various administra- tions. In Group 1-3, rats were orally administered with 4, 20 and 100 mg·kg⁻¹·d⁻¹ AH (dissolved in physiological saline), respectively. In Group 4 (control group), rats were orally administered with isopyknic physiological saline. After oral administration once a day for 7 consecutive days, rats were sacrificed 1 hr after the last treatment. The liver was immediately removed and stored in liquid nitrogen. Part of the liver of each rat was used to prepare rat liver microsome (RLM) by CaCl₂ precipitation method (Peng et al., 2009), and the protein concentration of RLM was determined by BCA Protein Assay Kit. The other part of the liver was used to measure the levels and activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), alanine transaminase (AST), aspartate transaminase (ALT), glutathione (GSH) and malondialdehyde (MDA).

Biochemical analysis for SOD, CAT, GSH-Px, AST, ALT, GSH and MDA
The liver homogenate was prepared in ice-chilled physiological saline, and was used to measure the levels and activities of SOD, CAT, GSH-Px, AST, ALT, GSH and MDA by the enzymatic reaction method using the commer- cially available kits (Nanjing Jiancheng Bioengineering Institute; Jiangsu, China).

Incubation in rat and human liver microsome
All incubations were performed at 37°C in 0.1 M potas- sium phosphate buffer (pH 7.4) containing 5 mM MgCl₂, probe substrates, RLM or HLM, 1 mM NADPH and AH (0 μM in RLM, 0-160 μM in HLM). The concentration of the probe substrate used for rat and human CYP isoforms in this work was chosen either at or near the apparent Km values of each rat or human CYP isoforms (Bjornsson et al., 2003) and displayed in Table 1. The concentration of microsomal protein (C) and incubation time (T) of each probe substrate was also displayed in Table 1. After pre- incubation (without NADPH in incubation medium) at 37°C for 3 min, the incubation reaction was started by the addition of NADPH, and terminated by the addition of 2-fold volume ice-cold methanol for testosterone, tolbuta- mide and phenacetin, 2-fold volume ice-cold ethyl acetate for dextromethorphan, bupropion and chlorzoxazone. The sample was then vortexed and centrifuged at 12,000 g for 10 min. The supernatant was evaporated by freeze- drying. The residue was re-dissolved in mobile phase and centrifuged at 12,000 g for 10 min. The supernatant was used for the content analysis of metabolite by HPLC or LC-MS/MS methods.

Fig. 1. The chemical structure of arecoline.
Determination of CYP activities in RLM and HLM

The metabolite production of the probe substrate in RLM and HLM incubation medium was used to evaluate CYP activity. Activity was calculated as the amount (moles) of metabolite formed per mg protein per minute incubation time. 6β-hydroxytestosterone, 4-hydroxytolbutamide, 6-hydroxychlorzoxazone and acetaminophen were determined by previously published HPLC methods (Chen et al., 2012; Guo et al., 2014) with slight modification. Dextrorphan and hydroxybupropion were determined by previously published LC-MS/MS method (He et al., 2007; Yeniceli et al., 2011) with slight modification. All of the analytical methods employed in the present work were validated according to accepted guidelines (Taverniers et al., 2004) (results not shown).

Results

Results are presented as mean ± S.D., and the comparison between groups was performed by one-way ANOVA, followed by paired-sample T-test to compare the mean values between the control group and drug treatment group. $P < 0.05$ was considered statistically significant.

### RESULTS

#### In vivo effect of AH on rat hepatic CYPs activity

The effect of oral administration of AH to rats on the activities of CYPs in RLM is displayed in Table 2. Oral administration of AH at the dose of 4 mg/kg/d resulted in 1.69-, 1.66-, 1.65-, 1.60-, 1.58- and 1.17-fold increase in the activities of CYP2B, 2E1, 2D, 3A, 2C and 1A2 in RLM, respectively. With the increase of oral dose of AH, the induction for the activities of CYP2B, 2E1, 2D, 3A and 2C was reduced, whereas for CYP1A2 activity was increased.

#### In vitro effect of AH on CYP activity in HLM

The in vitro effect of AH on the activities of CYPs in HLM is displayed in Fig. 2. Compared to the control (without AH in incubation system), AH slightly inhibited the activities of CYP3A4, 2D6, 2B6, 2E1, 1A2 and 2C9 in HLM, and the inhibition was found to increase with increasing concentration of AH. At concentrations up to 160 μM, the activities of CYP3A4, 2D6, 2B6, 2E1, 1A2

### Table 1 The incubation conditions in RLM and HLM

<table>
<thead>
<tr>
<th>CYP isoforms (RLM/HLM)</th>
<th>Probe substrate (μM) (RLM/HLM)</th>
<th>Metabolite (RLM/HLM)</th>
<th>C (mg/ml) (RLM/HLM)</th>
<th>T (min) (RLM/HLM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2/1A2 Phenacetion (50/50)</td>
<td>Acetaminophen</td>
<td>0.5/0.75</td>
<td>20/45</td>
<td></td>
</tr>
<tr>
<td>2E1/2E1 Chlorzoxazone (75/75)</td>
<td>6-hydroxychlorzoxazone</td>
<td>1.0/0.5</td>
<td>40/60</td>
<td></td>
</tr>
<tr>
<td>3A/3A4 Testosterone (250/100)</td>
<td>6β-hydroxytestosterone</td>
<td>0.5/0.5</td>
<td>10/60</td>
<td></td>
</tr>
<tr>
<td>2C/2C9 Tolbutamide (250/150)</td>
<td>4-hydroxytolbutamide</td>
<td>0.5/1.0</td>
<td>30/45</td>
<td></td>
</tr>
<tr>
<td>2D/2D6 Dextromethorphan (2.5/5)</td>
<td>Dextrorphan</td>
<td>0.25/0.5</td>
<td>25/25</td>
<td></td>
</tr>
<tr>
<td>2B/2B Bupropion (75/75)</td>
<td>Hydroxybupropion</td>
<td>0.5/0.25</td>
<td>20/20</td>
<td></td>
</tr>
</tbody>
</table>

Note: C (mg/ml) – the concentration of microsomal protein; T (min) – the incubation time

### Table 2 The effects of oral administration of AH on hepatic CYP activity in RLM

<table>
<thead>
<tr>
<th>Oral dose of AH (mg/kg/d)</th>
<th>CYP 2B</th>
<th>CYP 2C</th>
<th>CYP 1A2</th>
<th>CYP 2E1</th>
<th>CYP 3A</th>
<th>CYP 2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0 ± 25.7</td>
<td>100.0 ± 15.2</td>
<td>100.0 ± 16.0</td>
<td>100.0 ± 11.1</td>
<td>100.0 ± 15.7</td>
<td>100.0 ± 21.5</td>
</tr>
<tr>
<td>4</td>
<td>169.5 ± 23.8**</td>
<td>158.7 ± 32.5**</td>
<td>117.7 ± 10.0*</td>
<td>166.0 ± 16.3**</td>
<td>160.7 ± 36.2**</td>
<td>165.8 ± 22.1**</td>
</tr>
<tr>
<td>20</td>
<td>151.4 ± 11.2**</td>
<td>135.5 ± 22.8**</td>
<td>134.7 ± 11.6**</td>
<td>122.6 ± 13.8*</td>
<td>147.5 ± 20.6**</td>
<td>153.3 ± 36.2*</td>
</tr>
<tr>
<td>100</td>
<td>121.6 ± 12.3</td>
<td>95.8 ± 33.5</td>
<td>161.8 ± 22.4**</td>
<td>109.5 ± 10.6</td>
<td>127.6 ± 27.2</td>
<td>130.5 ± 21.9*</td>
</tr>
</tbody>
</table>

n = 6, mean ± S.D.

*P < 0.05 vs control group.

**P < 0.01.
and 2C9 in HLM were reduced by 17.6%, 36.2%, 38.3%, 35.0%, 14.6% and 35.4%, respectively. The IC_{50} values of AH on the six tested CYP isoforms are calculated using GrapPad Prism 5 software (GrapPad Co. Ltd., La Jolla, CA, USA) by non-linear regression, and are all greater than 160 μM.

**In vivo** effect of AH on hepatic oxidative stress and liver injury

The effect of oral administration of AH on the activities of SOD, CAT, GSH-Px, AST and ALT, as well as the levels of GSH and MDA in liver of rats are displayed in Table 3. Compared to the control, oral administration of AH to rats resulted in a decrease in the activities of SOD, CAT, GSH-Px and the level of GSH, whereas resulted in an increase in the activities of AST and ALT and the level of MAD, in dose-dependent manner. However, there are no statistically significant differences in the low dose for the effects on the hepatic activities of SOD, CAT, GSH-Px, ALT and AST, in the medium dose for the effects on the hepatic activities of CAT and GSH-Px.

**DISCUSSION**

Oxidative stress, initiated by reactive oxygen species (ROS), is the collective pathophysiological mechanism of many kinds of hepatopathy. It is known that thiol depletion as well as ROS attack should be mainly responsible for arecoline-induced hepatotoxicity (Dasgupta et al., 2006; Chang et al., 2001), and CYP enzymes play critical roles in mutagenic activation of the areca-specific N-nitrosamines (Miyazaki et al., 2005). The present work found that AH (4 and 20 mg/kg/d) significantly increased the hepatic CYP2E1 activity. CYP2E1 induction results in the generation of ROS through the reduction of molecular oxygen to water by NADH- and NADPH-dependent processes, and the ROS release induced by CYP2E1 is believed to play an important role in hepatic oxidative damage (Kathivel et al., 2010; Liu et al., 2005). Because the characteristics of AH exposure in AH recipient or betel-quid use human is always low dose and long period. So, the induction of AH on hepatic CYP2E1 activity should be an important mechanism of AH-induced ROS generation and oxidative damage in rat liver. On the other hand, AH (4 and 20 mg/kg/d) also significantly increased the hepatic CYP2B activity. CYP2B induction is asso-
Effects of arecoline on CYP450s and OS

### Table 3  The effects of oral administration of AH on hepatic oxidative stress

<table>
<thead>
<tr>
<th>Oral dose of AH (mg/kg/d)</th>
<th>MDA (nmol/mgprot)</th>
<th>SOD (U/mgprot)</th>
<th>CAT (U/mgprot)</th>
<th>GSH-Px (U/mgprot)</th>
<th>GSH (μg/mgprot)</th>
<th>ALT (U/gprot)</th>
<th>AST (U/gprot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.42 ± 0.08</td>
<td>141.9 ± 11.3</td>
<td>75.4 ± 8.7</td>
<td>1848.1 ± 91.5</td>
<td>10.9 ± 1.6</td>
<td>84.8 ± 6.6</td>
<td>90.5 ± 6.5</td>
</tr>
<tr>
<td>4</td>
<td>0.59 ± 0.10**</td>
<td>130.5 ± 2.45</td>
<td>76.0 ± 4.9</td>
<td>1804.7 ± 27.9</td>
<td>8.4 ± 1.3*</td>
<td>89.5 ± 6.8</td>
<td>97.8 ± 9.9</td>
</tr>
<tr>
<td>20</td>
<td>0.73 ± 0.21**</td>
<td>126.0 ± 10.9*</td>
<td>66.8 ± 9.0</td>
<td>1770.1 ± 26.3</td>
<td>7.5 ± 1.6**</td>
<td>92.2 ± 9.5*</td>
<td>108.1 ± 10.7**</td>
</tr>
<tr>
<td>100</td>
<td>1.08 ± 0.29**</td>
<td>120.5 ± 10.6**</td>
<td>61.2 ± 4.9*</td>
<td>1735.8 ± 25.9*</td>
<td>7.0 ± 1.2**</td>
<td>95.1 ± 7.5*</td>
<td>113.4 ± 9.5**</td>
</tr>
</tbody>
</table>

n = 6, mean ± S.D.
*P < 0.05 vs control group.
**P < 0.01.

The in vitro and in vivo effects of isolated active herbal constituents on the hepatic drug-metabolizing enzymes are useful for prediction of possible metabolic interaction (Bjornsson et al., 2003; Subehan et al., 2008; Zhao et al., 2012). It was reported that arecoline treatment increased the hepatic levels of Cyt b5 and CYP450, but had no obvious effect on the hepatic levels of GST and sulhydr-yl in lactating and suckling mice (Sing et al., 2000). Arecoline treatment also inhibited TCDD-induced CYP1A1 activation by down-regulation of AhR expression, but had no direct effect on CYP1A1 activity in human hepatoma cells, suggesting the possible involvement of arecoline in the AhR-mediated metabolism of environmental toxicants in liver (Chang et al., 2007). The present work found that the in vitro IC_{50} values of AH on the selected six kinds of human CYP isoforms in HLM were all greater than 160 μM, and these results indicated that AH should be a weak or non-inhibitor of the tested human CYP isoforms in vitro according to the literature (Cloe, 2010). However, oral administration of AH, especially at low dose, resulted in a significant increase for the activities of CYP2B1, 2D1, 3A, 2C and 1A2 in rat liver. It is known that the in vivo effect of CYP activity is more important for prediction of possible metabolic interaction than the in vitro effect. The in vivo induction of AH on rat CYP isoforms strongly suggested that the high risk of metabolic interaction should be existed when the substrate drugs of the six kinds of CYP isoforms was administered in arecoline recipient or betel-quid use human.

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