Author's response to reviews

Title: Up-regulation on Cytochromes P450 in Rat Mediated by Total Alkaloid Extract from Corydalis yanhusuo

Authors:

Jingjing Yan (yanjing5334@126.com)
Xin He (hexintn@163.com)
Shan Feng (374843695@qq.com)
Yiran Zhai (1102747372@qq.com)
Yetao Ma (370072650@qq.com)
Sheng Liang (747533213@qq.com)
Chunhuan Jin (420234936@qq.com)

Version: 3
Date: 6 June 2014

Author's response to reviews: see over
Subject: MS: 1686274756122430
Title: Up-regulation on Cytochromes P450 in Rat Mediated by Total Alkaloid Extract from *Corydalis yanhusuo*

Dear Sir,

We are extremely thankful for the invaluable comments we have received from reviewers who have generously shared their precious time and professional expertise to help us improve this paper. We are even more grateful to the reviewer for his insightful scientific suggestions to help make the manuscript more suitable for publication in *BMC Complementary and Alternative Medicine*. We have therefore revised the manuscript in keeping with the reviewers’ comments below. Taking all your comments into account, we have revised the manuscript accordingly and labeled them in red color. Our responses to each comment are given one after the other as raised by the reviewers.

Once again thanks for your consideration.

Yours sincerely,

Xin He, Ph.D.
Professor & Director
School of Chinese Materia Medica,
Tianjin University of Traditional Chinese Medicine,
312 Anshanxi Road, Nankai District,
Tianjin 300193, P.R. China.
Tel.: +86-22-5959-6231;
Fax: +86-22-5959-6153;
E-mail: hexintn@163.com
Reviewer: Shingen Misaka

Comments:

1. Method, Sample preparation and analysis - Please check whether the mobile phase (A) and (B) are correctly described. Generally, in the reverse-phase HPLC, the ratio of the “organic solvent” should be increased over time to elute the retained hydrophobic compounds.

Response: thanks for your advice. We have already corrected this mistake.

P5 as follow:

The mobile phase were (A) 0.5% phosphate buffer (pH 5.0) and (B) methanol and the elution system was as follows: 0 - 25 min, from 85% A to 70% A; 25 - 75 min, from 70% A to 65% A; 75 - 103 min, from 65% A to 25% A; 103 - 105 min, from 25% A to 20% A; 105 - 115 min, from 20% A to 85% A; 115 - 125 min, 85% A.

2. Method, Sample preparation and analysis, In 18-19 - Please state the definition of %, such as (w/w).

Response: thanks for your advice. We have already stated the definition of % (w/w).

P5 as follow:

The major alkaloids in YHS extract were quantified, and HPLC (Fig. 1) showed that the contents (w/w) of protopine, allocryptopin, berberine, dehydrocorydaline, tetrahydropalmatine, corydaline, tetrahydroberberine, and glaucine were 2.52%, 1.67%, 5.80%, 0.34%, 3.58%, 3.0%, 0.22%, and 0.66%, respectively.

3. Method, Microsomal CYPs activity detection - Please add detailed information about LC/MS/MS method, e.g. the apparatus used, ionization mode, mobile phase and m/z of each target compound.

Response: thanks for your advice. We have already added detailed information about LC/MS/MS method.

P7 as follow:

The HPLC system consisted of an LC-20AD pump, a DGU-20 A3 degasser, an SIL-20AC autosampler and a CTO-20A column oven (Shimadzu, Japan). The
Separation was performed on an Agilent ZORBAX XDB-C18 column (50 mm × 2.1 mm × 3.5 µm, Agilent). The flow rate was 0.45 mL/min and consisted of water with 0.1% formic acid (A) and methonal with 0.1% formic acid (B) using a gradient elution (0.0 - 0.5 min, 98% A; 0.5 - 1.0 min, from 98% A to 2% A; 1.0 - 2.5 min, 2% A; 2.5 - 2.51 min, from 2% A to 98% A; 2.51 - 4.0 min, 98% A) were used for analysis. An API 4000 Qtrap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with electrospray ion (ESI) source was used for mass analysis and detection. Following optimization of the setting parameters, the ESI source was operated in both positive mode (PAR, DEXP, OHMDZ, OHTOL, and Kamaxiping) and negative mode for (OHCHL) with the curtain, nebulizer and turbo-gas (all nitrogen) set at 15, 60 and 55 psi, respectively. The source temperature was 550°C and the ion spray needle voltage was 5000 V in positive mode and -4200 V in negative mode. PHE (CYP1A2), TOL (CYP2C11), DEXM (CYP2D1), CHL (CYP2E1), and MDZ (CYP3A1) were selected as the CYPs isoform probe substrates for the current study. The multiple reaction monitoring (MRM) mode was chosen for quantification of the metabolites of the probe substrates (Table 1).

Table 1. MRM transitions and collision energies for the detection of CYPs probe substrate metabolites

<table>
<thead>
<tr>
<th>Substrate metabolite</th>
<th>Molecular mass (MW)</th>
<th>Precursor (m/z)</th>
<th>Product (m/z)</th>
<th>Polarity</th>
<th>Collision Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol (PAR)</td>
<td>151</td>
<td>152</td>
<td>110</td>
<td>ESI⁺</td>
<td>23</td>
</tr>
<tr>
<td>Dextrophan (DEXP)</td>
<td>257</td>
<td>258</td>
<td>199</td>
<td>ESI⁺</td>
<td>38</td>
</tr>
<tr>
<td>1-Hydroxymidazolam (OHMDZ)</td>
<td>341</td>
<td>342</td>
<td>203</td>
<td>ESI⁺</td>
<td>40</td>
</tr>
<tr>
<td>4-Hydroxytolbutamide (OHTOL)</td>
<td>286</td>
<td>287</td>
<td>171</td>
<td>ESI⁺</td>
<td>59</td>
</tr>
<tr>
<td>6-hydroxychlorzoxazone (OHCHL)</td>
<td>185</td>
<td>184</td>
<td>120</td>
<td>ESI⁻</td>
<td>-26</td>
</tr>
<tr>
<td>Kamaxiping (internal standard)</td>
<td>236</td>
<td>237</td>
<td>194</td>
<td>ESI⁺</td>
<td>40</td>
</tr>
</tbody>
</table>

4. Method, Statistical analysis - Please provide the post-hoc test performed for the multiple comparison following one-way ANOVA.

Response: thanks for your advice. We have already corrected the description of statistical analysis method. Actually, independent-sample t-test was used to analyze
the differences between the enzyme activities, gene expression values with control values by SPSS 13.0.

P8 as follow:

Independent-sample t-test was used to analyze the differences between the enzyme activities, gene expression values with control values by SPSS 13.0. Differences were considered significant at $P < 0.05$.

5. Results, pg 8, ln 13 and ln 16-“62.8%” should be 68.2%. Also, “CYP2C9” should be CYP2C11.
Response: thanks for your advice. We have already corrected this mistake. TAE dosages (30mg/kg, daily) significantly increased the enzyme activity of CYP2E1 by 68.2%. The highest TAE dosage (150mg/kg, daily) increased the enzyme activity of CYP2C11 by 35.4%.

6. Table 2 - Was there no statistically significance between values of control and low dose TAE (6 mg/kg) in CYP3A1 activity? Because the respective SD values seem low sufficient to reach a significance level.
Response: thanks for your advice. We have already corrected this mistake. Independent-sample t-test was used to analyze the differences between the enzyme activities again, the result showed that there was statistically significance between values of control and low dose TAE (6 mg/kg) in CYP3A1 activity.

P9 as follow:

In the treated rats, the three TAE dosages (6, 30, and 150 mg/kg, daily) significantly increased the enzyme activity of CYP2E1 by 20%, 68.2%, and 146.1%, respectively, and the three TAE dosages increased the levels of CYP3A1 by 27.84%, 40.3% and 51.9%, respectively, compared with those in the control rats.

7. Discussion, pg 9, ln 27 and ln 35-move qi” may be a typo. “CYP3A4” should be CYP3A1.
Response: thanks for your advice. We have already corrected this mistake.
8. Discussion—There is no discussion about the effect of TAE from YHS on CYP2D1. Although TAE did not affect CYP2D1 mRNA level and activity, this finding may also be important in terms of safety use of YHS.

Response: thanks for your advice. We have made a further discussion on P12-13 about the effect of TAE from YHS on CYP2D1 and the research status of CYP2D1.

P12-13 as follow:

After the treatment of rats for 14 days with different dosages of TAE, significant increases were observed in the mRNA expression and enzyme activities of CYP1A2 and CYP2C11 at 150 mg/kg TAE, but the mRNA levels and enzyme activities of CYP2D1 did not change significantly among the three TAE dosages. In the human liver, CYP1A2, CYP2C and CYP2D6 are involved in the metabolism of 4%, 16%, and 30%, respectively, of drugs on the market [11].

CYP2D1 is the rat orthologue of human CYP2D6, and CYP2D6 has been the most studied human genetic polymorphism in drug metabolism [11]. In vivo clearance of CYP2D6 substrates in poor metabolizers is generally much lower than in extensive metabolizers, leading to higher plasma concentrations and the potential for clinical toxicities with therapeutic doses [40].

And TAE did not affect CYP2D1 mRNA level and activity in three doses, the finding suggests that the use of products containing YHS may be considered safe when co-administration with CYP2D1 substrate, inhibitor, and inducer.

9. Figure 1—Compared with the retention time of other peaks, there is a larger difference (2-3 min) in the retention time of the peak 3 (berberine) between the real sample and the reference standard. How to identify that the peak 3 in the real sample is berberine.

Response: thanks for your advice. We added the Standard of berberine in the TAE from YHS, and we have identify that the peak 3 in the real sample not the berberine. Fig.1-1 showed that berberine is not in the sample of TAE from YHS. So we have corrected our results in our article.
Fig.1-1, A: Chromatogram of the total alkaloid extract (TAE) from YHS and berberine; B: chromatogram of reference standards.

P5 as follow:

The major alkaloids in YHS extract were quantified, and HPLC (Fig. 1) showed that the contents (w/w) of protopine, allocryptopin, berberine, dehydrocorydaline, tetrahydropalmatine, corydaline, tetrahydroberberine, and glaucine were 2.52%, 1.67%, 5.80%, 0.34%, 3.58%, 3.0%, 0.22%, and 0.66%, respectively. Figure 2 presents the structures of the alkaloid components.
Fig. 1. HPLC-fingerprint chromatograms of total alkaloids of YHS. (A) Chromatogram of the total alkaloid extract (TAE); (B) chromatogram of reference standards; and (C) chromatogram of the blank solvent; 1, protopine; 2, allocryptopin; 3, dehydrocorydaline; 4, tetrahydropalmatine; 5, corydaline; 6, tetrahydroberberine; 7, glaucine.

Fig. 2. Chemical Structure of the main alkaloid components of YHS.
Reviewer: LIK VOON KIEW

Comments:

1. Please provide the reference number for the ethics approval granted by the institutional animal care and use committee (IACUC). i.e. in this case, the Tianjin University of Traditional Chinese Medicine Animal Ethics Committee.

Response: thanks for your advice. We have provided the reference number for the ethics approval granted by the institutional animal care and use committee (IACUC).

All procedures involving animals were conducted in conformity with the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines, and were approved by the Academy of Military Medical Science Institutional Animal Care and Use Committee (Certificate No. SCXK620076004).

2. a. The information on the number of animal per treatment groups (n number) was not found.

Response: thanks for your advice. We have made an information on the number of animal per treatment groups.

Rats were randomly divided into 5 groups (total 50 rats, n = 10): three TAE-treated groups, the control group, and the positive control group, respectively.

2.b. 2 weeks oral administration of TAE: no information on the frequency of the administration / time interval in between administration.

Response: thanks for your advice. We have made a information on the frequency of the administration / time interval in between administration.

In 14 consecutive days, TAE-treated groups were given TAE orally at doses of 6, 30, 150 mg/kg once daily, the positive control group was intraperitoneal injection with phenobarbital (80mg/kg) once daily [16], whereas the control group was orally with equivalent 0.5% sodium carboxymethylcellulose solution once daily.
2.c. The test compounds were orally administered while the positive control was administered i.p. Please provide justification / reference for the use of i.p. injection method for the administration of the positive control compound.

Response: thanks for your advice. We have found a reference for the use of i.p. injection method for the administration of the positive control compound. We have added it in our reference.

P16 as follow:


2.d. CMC control group: mode of administration missing.

Response: thanks for your advice. We have added the mode of administration of CMC control group on P6.

2.e. Details on the rat sacrificial procedure and blood sampling are not provided (required for considerations in terms of animal ethics and possible drug interaction if anaesthetics are used).

Response: thanks for your advice. We have given the details on the rat sacrificial procedure and blood sampling on P6.

P6 as follow:

After two weeks, the rats were quickly anaesthetized with urethane and then sacrificed by exsanguination of aorta abdominalis. The blood samples were centrifuged at 10000 g for 5 min at 4℃. Serum was collected immediately after each spin and stored at -80℃ until the time of assay.

2.f. “the dosage of 30 mg/kg ⋮”: is this sentence referred to the TAE dose?

Response: thanks for your advice. 30 mg/kg is the TAE dose. We have corrected it on P6.
3. a. “0/100/2.5/20/5 \mu M PHE/TOL/DEXM/CHL/MDZ”: please specify the purpose of these substrates (please correlate them to respective CYPs monitored).

Response: thanks for your advice. We have already specified the purpose of these substrates on P7.

P7 as follow:

PHE (CYP1A2), TOL (CYP2C11), DEXM (CYP2D1), CHL (CYP2E1), and MDZ (CYP3A1) were selected as the CYPs isoform probe substrates for the current study.

3. b. Information on the LC-MS/MS setup/condition are not available.

Response: thanks for your advice. We have already given the information on the LC-MS/MS condition.

P7 as follow:

The HPLC system consisted of an LC-20AD pump, a DGU-20 A3 degasser, an SIL-20AC autosampler and a CTO-20A column oven (Shimadzu, Japan). The separation was performed on an Agilent ZORBAX XDB-C18 column (50 mm × 2.1mm × 3.5 µm, Agilent). The flow rate was 0.45 mL/min and consisted of water with 0.1% formic acid (A) and methonal with 0.1% formic acid (B) using a gradient elution (0.0 - 0.5 min, 98% A; 0.5 - 1.0 min, from 98% A to 2% A; 1.0 - 2.5min, 2% A; 2.5 - 2.51 min, from 2% A to 98% A; 2.51 - 4.0 min, 98% A) were used for analysis. An API 4000 Qtrap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with electrospray ion (ESI) source was used for mass analysis and detection. Following optimization of the setting parameters, the ESI source was operated in both positive mode for (PAR, DEXP, OHMDZ, OHTOL, and Kamaxiping) and negative mode for (OHCHL) with the curtain, nebulizer and turbo-gas (all nitrogen) set at 15, 60 and 55 psi, respectively. The source temperature was 550°C and the ion spray needle voltage was 5000 V in positive mode and -4200 V in negative mode. PHE (CYP1A2), TOL (CYP2C11), DEXM (CYP2D1), CHL (CYP2E1), and MDZ (CYP3A1) were selected as the CYPs isoform probe substrates for the current study.
study. The multiple reaction monitoring (MRM) mode was chosen for quantification of the metabolites of the probe substrates (Table 1).

### Table 1. MRM transitions and collision energies for the detection of CYPs probe substrate metabolites

<table>
<thead>
<tr>
<th>Substrate metabolite</th>
<th>Molecular mass (MW)</th>
<th>Precursor (m/z)</th>
<th>Product (m/z)</th>
<th>Polarity</th>
<th>Collision Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol (PAR)</td>
<td>151</td>
<td>152</td>
<td>110</td>
<td>+ESI</td>
<td>23</td>
</tr>
<tr>
<td>Dextrophan (DEXP)</td>
<td>257</td>
<td>258</td>
<td>199</td>
<td>+ESI</td>
<td>38</td>
</tr>
<tr>
<td>1-Hydroxymidazolam (OHMDZ)</td>
<td>341</td>
<td>342</td>
<td>203</td>
<td>+ESI</td>
<td>40</td>
</tr>
<tr>
<td>4-Hydroxytolbutamide (OHTOL)</td>
<td>286</td>
<td>287</td>
<td>171</td>
<td>+ESI</td>
<td>59</td>
</tr>
<tr>
<td>6-hydroxychlorzoxazone (OHCHL)</td>
<td>185</td>
<td>184</td>
<td>120</td>
<td>+ESI</td>
<td>-26</td>
</tr>
<tr>
<td>Kamaxiping (internal standard)</td>
<td>236</td>
<td>237</td>
<td>194</td>
<td>+ESI</td>
<td>40</td>
</tr>
</tbody>
</table>

3.c. Information on the methods and results for the recovery (extraction efficiency) of analytes are not available.

Response: thanks for your advice. We have already given the information on the methods and results for the recovery (extraction efficiency) of analytes.

P7 as follow:

The recovery (extraction efficiency) of PAR, OHTOL, DEXP, OHCHL, and OHMDZ from HLMs were determined at three different concentrations of quality control samples (they were 0.1, 0.5, and 2.5 µM). The internal standard was carbamazepine (75 ng/ml). The extraction recoveries of PAR was 86.31% ± 2.01, OHTOL was 92.14% ± 1.57, DEXP was 87.19% ± 1.33, OHCHL was 93.34% ± 1.97, and OHMDZ was 90.76% ± 1.87.

3.d. Information related to the translation LC-MS/MS data to enzyme activity are not provided.

Response: thanks for your advice. We have already given the information related to the translation LC-MS/MS data to enzyme activity.

P8 as follow:

The enzyme activity was calculated as follows: enzyme activity = C_{metabolite} × L_{incubation}/T_{incubation} / (C_{HLMs} × L_{incubation}), in which C_{metabolites} represents the concentration
of metabolites of CYPs probe substrates, $L_{\text{incubation}}$ represents the volume of incubation system, $T_{\text{incubation}}$ represents the incubation time, and $C_{\text{HLMs}}$ represents the concentration of HLMs. Values were expressed in units of pmol $\cdot$ min$^{-1} \cdot$ mg$^{-1}$.

4. The amount of tissue subjected for processing and the method/procedure of tissue processing (particularly the intestine) was not provided.

Response: thanks for your advice. We have already provided the information about the amount of tissue subjected for processing and the method of tissue processing on P6 and P8.

P6 and P8 as follow:

Livers, kidneys, lungs and intestines were removed immediately and rinsed with physiological saline. Liver was cut into small pieces to get the liver tissue. Renal cortex was removed and minced in slices to get the kidneys tissue. Lung was cut into small pieces to get the lung tissue. To get the intestinal mucosa, pieces of intestine were placed on an ice-cold glass plate, and the intestinal mucosa gently squeezed out. All the tissues were stored at -80°C for further analysis.

Total RNA were extracted from the samples of liver (approximately 50 mg), kidney (approximately 50 mg), lung (approximately 50 mg) and intestine (approximately 50 mg) were extracted using Trizol reagent (Applied Biosystems, Foster City, CA, USA) according to the protocol provided by the manufacturer.

5. Statistical analysis-Please specify post-hoc test employed for one-way ANOVA..

Response: thanks for your advice. We have already corrected the description of statistical analysis method. Actually, independent-sample t-test was used to analyze the differences between the enzyme activities, gene expression values with control values by SPSS 13.0.

P8 as follow:

Independent-sample t-test was used to analyze the differences between the enzyme activities, gene expression values with control values by SPSS 13.0. Differences were considered significant at $P < 0.05$. 
Discussion section:
CYP2E1 induction associated with TAE toxicity
Paragraph 4:
“The liver injury caused by YHS may thus have resulted from the induction of the drug metabolic enzyme CYP2E1 by long-term administration of YHS or from its metabolism by CYP2E1 to a toxic metabolite.”: please provide examples for the “toxic metabolite”.
Response: thanks for your advice. The mechanism of toxicity research of YHS is limited. At first, I think the reason that the liver injury caused by YHS may be YHS can be metabolized by CYPs to a toxic metabolism. The sentence of “…from its metabolism by CYP2E1 to a toxic metabolite” was not rigorous, so I deleted the sentence on P11.

Induction ability of TAE on CYP3A1 in rats
As mentioned previously, rat may not be a good model of liver microsomal metabolism dependent on CYP3A4. The current discussion on the elevation of CYP3A1 and the indication on the risk of drug-drug interaction will be meaningful if the author can provide some examples of the clinically important drugs that are metabolised by both the rat CYP3A1 and human CYP3A4 (with relevant references), or provide references on the CYP3A1 metabolism of drug examples mentioned in the discussion.
Response: thanks for your advice. We have provided references on the CYP3A1 metabolism of drug examples on P12.

P12 as follow:

CYP3A1 can catalyse the 6β-hydroxylation of testosterone [33] and the metabolism of a large variety of clinical medications, including many pediatric drugs [34], cyclosporin A [35].

7. RT-PCR
a. Possible typo error in line 1: “RAN”.

Response: thanks for your advice. We have already corrected this mistake.

Discussion section:
The influence of TAE on other CYPs
Rats CYP2C enzymes, which is the most abundant CYP subfamily of rat liver has been previously suggested to have a role of human CYP3A enzyme. Since the current results showed that TAE only induces CYP2C11 at high concentration, the author may want to consider to re-examine the indication of the current results, particularly on its correlation to the activity of the human CYP3A enzyme.

Response: Thanks for you give the information that rat CYP2C have a role of human CYP3A enzyme. Animal models are commonly used in the preclinical development of new drugs to predict the metabolic behaviour of new compounds in human. It is however important to realize that humans differ from animals with regard to isoform composition, expression and catalytic activities of drug metabolizing enzymes. We know that CYP3A seems to be well modelled by pig or minipig CYP3A29 (Zuber et al. (2002)). Therfore, in next experiment, we can choose pig or minipig CYP3A29 to study TAE from YHS effect on CYP3A enzyme activity.