Author's response to reviews

Title: Study on the antiviral activity of San Huang Yi Gan Capsule against hepatitis B virus with seropharmacological method

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Author's response to reviews: see over
Dear Sir or Madam,

I would like to submit our revised manuscript entitled "Study on the antiviral activity of San Huang Yi Gan Capsule against hepatitis B virus with seropharmacological method" for your consideration for publication as a research article in the *BMC Complementary and Alternative Medicine*. We greatly appreciate the reviewers for their critical comments and constructive suggestions. We have provided our response to the reviewers’ comments below and believe our manuscript is much improved as a result. The full institutional names of the animal ethics committees that approved the study are included in the revised manuscript.

Many thanks in advance for your consideration of our manuscript for publication.

Best regards,

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Response to reviewers

Reviewer 1

Major points:

1. For quantitation of HBV DNA, the quantitative PCR would be considered as clinical proved assay method. If authors want to study the different forms of HBV, the northern blot would be considered as mechanistic study.

   We totally agree that quantitative PCR is another method to quantify the HBV DNA level. However, quantitative PCR was not taken in this study based on the following points. First, the dot blot hybridization is a more specific and reliable than quantitative PCR in measuring DNA level. Second, quantitative PCR is more likely to get false positive result and false negative result, compared to the dot blot hybridization. Third, determination of the level of HBV DNA affected by SHYGC-containing sera is just an example for seropharmacological study, ultimately proving the seropharmacological method is applicable and reliable in biomedical research. The main focus of our current work is not the thorough mechanism of SHYGC regulating HBV DNA, which obviously needs to be further addressed in another manuscript. Therefore, northern blot was not considered either. Fourth, quantitative PCR was, unfortunately, not easily accessible in our institutes at the time of this study.
2. The efficacy of positive control, Entecavir, did not show a good HBV viral DNA inhibition in this study. If this seropharmacology is applied to study unknown bioactive materials, the standard or control substance should be explained more in details for optimizing the assay method. Especially, authors point out seropharmacology could be connected the pharmacokinetic study for exploring active ingredient of traditional medicine or providing toxicological issue such as safety pharmacology.

Entecavir is an anti-HBV agent, approved by the FDA in March 2005, used in the chronic hepatitis B treatment. By competing with the natural substrate deoxyguanosine triphosphate, entecavir functionally inhibits all three activities of the HBV polymerase (reverse transcriptase, rt): (1) base priming, (2) reverse transcription of the negative strand from the pregenomic mRNA, and (3) synthesis of the positive strand of HBV DNA. In our study, entecavir was found to drastically inhibit HBV DNA polymerase activity and HBV DNA replication in HepG2 2.2.15 cells, consistent with other reports [1]. Although entecavir did not show significant effect on HBsAg expression, it is still a good positive control in this study.

Minor points:

1. Why did authors use the rabbits for seropharmacology study?

As stated in the discussion, rabbits were selected to prepare SHYGC-containing sera, because they can provide more sera than rats and mice. And, rabbits can be
obtained more easily, compared to other bigger experimental animals.

2. **How to refresh the drug serum-conditioned medium in the efficacy test?** For once or refresh it daily.

   We just added the drug sera-containing medium once and did not refresh the medium mixture during the cell culture for the efficacy test, in order to enrich the HBV in the supernatant medium to a higher concentration.

**References:**


**Reviewer 2**

On page 6: The San Huang Yi Gan Capsule (SHYGC) was dissolved at 500 mg/ml. The capsule containing 1.67g of medicinal plants. What is the nature of the plant materials, are they water soluble extract or mixture of whole plant powder? How can it be dissolved in water if it is powder mix? Was it suspended in water or dissolved in water?
San Huang Yi Gan Capsule (SHYGC) is composed of the water soluble extract of 6 Chinese herbs, so it is easily dissolved in double distilled water. We have addressed the composition of San Huang Yi Gan Capsule (SHYGC) in detail in the revised manuscript. There is no any other component in the capsule, and the ratio of the 6 Chinese herbs can not be disclosed currently, due to the intellectual property right issue.

“Entecavir (ETV), derived from Bristol-Myers Squibb Corporate (New York, NY), was dissolved in dimethyl sulfoxide at 10µM and prepared in cell culture medium prior to the following tests”. This sentence does not make sense. Was it dissolved in 100 % DMSO? Or in cell culture medium? Or 10% DMSO containing cell culture medium? Prior to following tests need to be changed as “prior to experimental test”.

The sentences have been reconstructed as the following: “Entecavir (ETV), derived from Bristol-Myers Squibb Corporate (New York, NY), was dissolved in 100% dimethyl sulfoxide at 10µM as a stock. Prior to experimental tests, ETV was diluted in cell culture medium, with working concentration of 10nM.” So, the final concentration of DMSO in the cell culture medium is 0.1%.

Page 7: “Rabbits of low, medium and high dose SHYGC groups orally received SHYGC in doses of 0.25g/kg, 0.5g/kg and 1.0g/kg daily, with rabbits of vehicle group administered the volume-matched ddH2O”. This sentence needed restructuring. Why placebo group control rabbit were fed matched volume ddH2O?
**H2O should be fed adlib. Does H2O contained DMSO also?**

The sentences have been reconstructed as the following: “Rabbits of low, medium and high dose SHYGC groups were gavaged SHYGC in doses of 0.25g/kg, 0.5g/kg and 1.0g/kg daily, while rabbits of vehicle group were gavaged the volume-matched ddH₂O.”

All rabbits were fed with standard diet and distilled water *ad libitum*, and there was no any DMSO in the ddH₂O. As the rabbits of SHYGC groups underwent the gavage, in order to keep the equal treatment in each group, the rabbits of vehicle group were gavaged the volume-matched ddH₂O.

**What is P/N ratio? P and N should be defined.**

HBsAg level was expressed as the value of sample (P) against negative control (N). This point has been clarified in the revised manuscript.

**Figure 1A: “Rabbit inactivated serum” should be stated as ‘inactivated rabbit serum’.**

We have corrected the error in the text. Many thanks for the reviewer’s help!

**Fig 1C: is not required. Why should there be any difference in blood serum withdrawn from ear artery or transcardial puncture?**

Some researchers prepare drug-containing serum via transcardial puncture, which is more harmful and stimulant to animal, and may possibly change the serum ingredients
(internal factors), compared to ear artery. We tried to demonstrate that drug-containing sera collected through the two approaches show no difference in pharmacological effect.

Fig 3. The authors have examined endogenous HBV DNA polymerase activity in serum treated medium supernatant. What is the direct effect of drug (if water soluble) on the polymerase activity in vitro?

In this study, we just found SHYGC inhibited the HBV DNA polymerase activity in a dose-dependent manner, while the in-depth mechanism is unknown, due to the limitation of the experiments. As entecavir functionally inhibits all three activities of the HBV polymerase (reverse transcriptase, rt): (1) base priming, (2) reverse transcription of the negative strand from the pregenomic mRNA, and (3) synthesis of the positive strand of HBV DNA [1]. We speculate that SHYGC may possibly act with some of the above mechanisms, which merit further investigation.

Page 13, second line from bottom: what is “prosperous strategy”? May be authors mean ‘proper strategy’.

We have amended the word in the text. Many thanks for the reviewer’s help!

Page 14 line 4 from bottom: “direction action” or direct action?

The “direction action” has been corrected as “direct action”. Many thanks for the reviewer’s help!
The scientific name of plants should be in italics with first letter of Genus in capital letter. Radix scutellariae, Rheum palmatum L., Radix bupleuri, Radix astragali, Fructus schizandrae and Radix glycyrrhizae.

We have corrected the errors in the revised manuscript. Many thanks for the reviewer’s help!

References: