July 17, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 11137 Manuscript revised.doc).

Title: Combination of Weichang'an with 5-fluorouracil Suppresses Colorectal Cancer in a Mouse Model

Author: Tao Li

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: MS ID 11137

The manuscript has been improved according to the suggestions of the Reviewers:

1 - Format has been updated

2 - Revision has been made according to the suggestions of the Reviewers

Reviewer #1

In this manuscript, Tao et al analyzed the therapeutic effect of WCA on colorectal cancer in an animal model. According to their results, WCA enhances the efficacy of 5-FU to inhibit colon cancer cells growth and hepatic metastases. They also describe the effect of WCA on the expression of the proteins beta-catenin and MMP-7, both of them involved in metastasis development in colorectal cancer. This in an interesting work addressing a very relevant topic in clinical gastroenterology. Authors used properly different techniques, qPCR, IHC and immunoblotting, in order to confirm their results about the effect of WCA on the expression of beta-catenin and MMP-7.

The manuscript is written in a good English and only some minor mistakes must be revised. However, I have some major and minor points that authors should address:

Major points：

Methods:

The description of Real time PCR is not clear. Moreover, authors must include the amplification efficiency values of each primer pair used for beta-catenin, MMP-7 and GAPDH amplification in order to confirm that those values are right. In addition, some authors have described that GAPDH is not the best reference gene for real time PCR in colorectal tissues. Authors must address this question.

Response: We thank the Reviewer for his comment. The real-time PCR section has been revised. We used the $2^{-\Delta\Delta Ct}$ method and the amplification efficiency values of each primer pair used for β-catenin, MMP-7 and GAPDH amplification were similar. We used β-actin and GAPDH in the preliminary experiment and the amplification efficiency of GAPDH was closer to that of the target genes, i.e. β-catenin and MMP-7. Therefore, we chose GAPDH as the reference gene.

Results:

Authors determine WCA concentration measuring hesperidin concentration. Is hesperidin the compound responsible for the therapeutic effect of WCA? In literature, there are reports describing hesperidin effect on proliferation in induced colon carcinogenesis in mice (Inflamm Res 2013, 62:425-40) as well as on apoptosis in human colon cancer cells (Phytomedicine 2008, 15:147-51). Authors must discuss this topic.

Response: Hesperidin is the major active component in WCA. To the best of our knowledge, hesperidin is a stable and controllable component in WCA. HPLC detection of hesperidin facilitates the monitoring of WCA concentration.

Hesperidin is a citrus flavonoid shown to be active against various oxidative stress-mediated diseases. Hesperidin found in orange peel is a flavanone glycoside consisting of the flavone hesperidin bound to the disaccharide rutinose. The sugar group makes hesperidin more water-soluble than hesperitin, another compound in

orange peel [1]. Hesperidin may moderately stimulate the gastrointestinal tract, promote the secretion of digestive enzymes, remove intestinal pneumatosis, and invigorate stomach to relieve phlegm. Exogenous hesperidin has been shown to influence a wide variety of biological functions. For example, hesperidin induces apoptosis and suppresses proliferation of human cancer cells [2], and inhibits tumor development in various tissues, including the colon cancer [3, 4]. Many of these beneficial effects of hesperidin can be attributed to its antioxidant activity [5]. In WCA, in cooperation with the other components, hesperidin functions as spleen invigorating, heat clearing, detoxicating and hard lump resolution.

We added some discussion about the effects of hesperidin.

[1] Vallejo F., Larrosa M., Escudero E., Zafrilla M. P., Cerda B., Boza J., Garcia-Conesa M. T., Espin J. C., Tomas-Barberan F. A. Concentration and solubility of flavanones in orange beverages affect their bioavailability in humans. *J. Agric. Food Chem.* (2010);58:6516–6524.

[2] Ghorbani A., Nazari M., Jeddi-Tehrani M., Zand H. The citrus flavonoid hesperidininduces p53 and inhibits NF-kappaB activation in order to trigger apoptosis in NALM-6 cells: involvement of PPARgamma-dependent mechanism. *Eur. J. Nutr*.(2012);51:39–46.

[3] Aranganathan S., Nalini N. Antiproliferative efficacy of hesperetin (citrus flavanoid) in 1,2-dimethyl hydrazine-induced colon cancer. Phytother Res. 2013 Jul;27(7):999-1005.

[4] Saiprasad G., Chitra P., Manikandan R., Sudhandiran G. Hesperidin alleviates oxidative stress and downregulates the expressions of proliferative and inflammatory markers in azoxymethane-induced experimental colon carcinogenesis in mice.*Inflamm. Res.* (2013);62:425–440.

[5] Elavarasan J., Velusamy P., Ganesan T., Ramakrishnan S. K., Rajasekaran D., Periandavan K. Hesperidin-mediated expression of Nrf2 and upregulation of antioxidant status in senescent rat heart. *J. Pharm. Pharmacol.* (2012);64:1472–1482.

Figure 4: Authors must indicate, as they do in figure 5, the statistical significance of their IHC results.

Response: Figure 4 was edited.

Discussion:

Authors must discuss the discrepant results about MMP-7 expression found by immunoblotting, IHC and real time PCR.

Response: In the present study, mRNA and protein levels of MMP-7 were down-regulated in orthotopic tumors and hepatic metastatic tumors after 5-FU, WCA or 5-FU+WCA treatments, as detected using immunoblotting, IHC and real-time PCR. The orthotopic transplant model used in this study is a relatively natural nude mouse model of hepatic metastases, and the metastasis rate is not very high. Only 3 to 5 animals in each group developed liver metastases, which is not very powerful on a statistical point of view. In a future study, we will increase the sample size and this problem may be resolved. We discussed the discrepant results about MMP-7 expression as a limitation of this study.

Minor points:

Introduction:

The paragraph "The principal element in WCA are……., previous clinical studies have indicated….. will benefit from WCA treatment " is not comprehensible. In my opinion, it must be split into 2 sentences.

Response: This was corrected.

Material and Methods:

For the statistical analysis considering the small number of mice included in each group, I would user non-parametric tests instead of the ANOVA test.

Response: We thank you for your suggestion. We re-analyzed the data of small mice number (3-5) using non-parametric tests. The Statistical analysis and Result sections

were revised.

Results:

Quality of figures must be improved. Figure 4: Authors must indicate, as they do in figure 5, the statistical significance of their IHC results

Response: Figure 4 was edited.

Reviewer #2

Tao et al. investigate the therapeutic efficacy of WCA in a mouse model of colorectal cancer. They find that WCA, alone or in combination with 5-FU has some effect on tumor growth an metastatic rate. The paper is well written and presented, and the findings might be interesting.

Please consider the following critical points:

1) Abstract: it is too descriptive, please add p values and other objective measures to back up your statements

Response: Abstract was revised and P-values were added.

2) Tab 1 and 2 are hard to read, since the titles of the columns are misplaced. Please correct

Response: Thank you for this comment. The tables have been reorganized.

3) Please indicate the statistical test employed after each p value, in the text and/or in the legends.

Response: This was corrected.

4) The most critical point is to show that WCA has an effect on colorectal cancer growth/metastasis, especially when added to 5FU. This point is not well investigated. The authors should directly compare 5FU and 5FU plus WCA in terms of growth rate, metastatic potential. Is there any significant advantage of adding WCA to classical chemotherapy?

Response: As we summarized in the abstract, 5-FU treatment alone significantly decreased tumor weight compared with the CON group. WCA treatment alone reduced the rate of metastasis. Combination treatment of WCA with 5-FU was particularly effective, reducing tumor weight and size, metastatic rate, and serum CEA levels. Considering the relatively less adverse effects of traditional Chinese medicine, the combination of WCA with 5-FU may present a good strategy in colon cancer treatment.

Reviewer #3

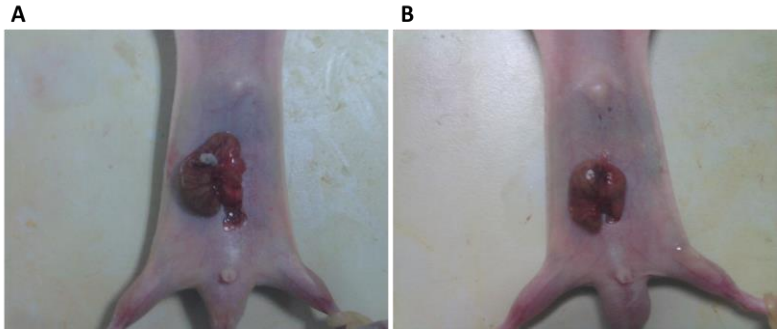
Tao et al. analyzed the effect of WCA, a traditional Chinese medicine on colorectal carcinoma in a clinically interesting orthotopic model. Their major finding was a reduction in tumor weight and size as well as the number of metastases when WCA was combined with 5-FU. They also speculate that a decreased expression of b-Catenin and MMP-7 may be involved in the anti-tumorigenic properties of WCA. The design, technical performance and some of the conclusions made in this study are comprehensible. The authors address a clinically very relevant topic. The manuscript is overall written in a very good English.

However, there are several major and minor points, the authors must address.

Major points:

1. The orthotopic implantation procedure was described – but since for this case, the technical procedure of the purse-string suture is not yet described in the literature, the authors should provide a more detailed description including some pictures. This may be of interest to many people working in the field.

Response: Purse-string suture pictures were added in Figure 2 (as below).



2. The phenomenon of lymphocyte infiltration in the nude mouse system is rather unexpected and we did not see this in comparable studies. What is the cell type? NK cells? B cells? Others?

Response: Lymphocyte infiltration and necrosis were observed in the tumor interstitium (Fig. 3), but we did not further analyze the cell types. We are preparing a study focusing on immunological effects of WCA in colorectal tumor model, in which these cells will be evaluated.

3. Honestly, claiming 100% tumor take with only 1/12 mice dying after such an invasive surgical procedure is hard to believe. Please provide reasons/data how you trained your mouse-surgeons to reach such a fantastic standard. Moreover, any disadvantages of the model used (as for example the use of a very old cell line of the compared to CIN rare molecular phenotype MSI; thus being most likely not an ideal model for 5-FU based therapies) are not at all addressed in the discussion. Finally, the fact that more mice died in the WCA groups is also not at all discussed.

Response: The gastric orthotopic transplantation tumor model has been used by our group for long time and we published several related works. The mouse surgeon is an experienced expert in animal experiments. In the present study, the tumor blocks were transplanted in cecum and purse string suture was performed. Occasionally, perforation and death occurred during the preliminary experiments, but the success rate greatly increased in later experiments.

One mouse in each group died within one week of orthotopic transplant surgery; one mouse died 12 days after surgery in the WCA group, and one mouse died 10 days after surgery in the WCA+5-FU group. According to our observations of the mice, we consider that their death may have been caused by the surgical trauma and subsequent infection, which needs further investigations. Therefore, we are planning an immunological study for the future.

The discussion section was revised according to your suggestion.

4. The claim: “Our findings suggest that WCA additively enhances the efficacy of 5-FU to inhibit colon cancer growth and metastasis” in the discussion is not supported by the data delivered, isn’t it?

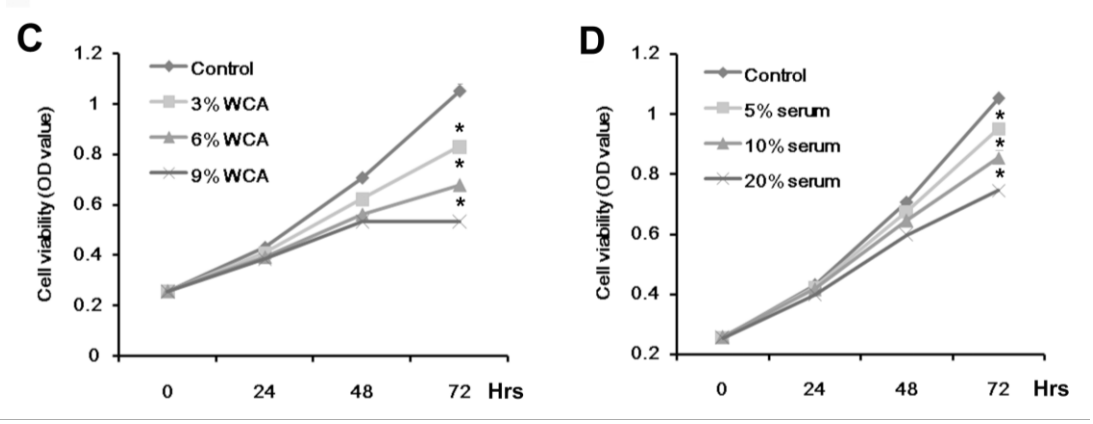
Response: We modified the conclusion as: ‘combination of traditional Chinese medicine WCA with chemotherapeutics 5-FU inhibits colon tumor growth and hepatic metastasis in this orthotopic transplant nude mouse model. Decreased expression of both β-Catenin and MMP-7 may be important for the anti-tumorigenic properties of WCA and 5-FU’.

5. The differences between the mRNA and W-Blot results versus immunohistochemistry for MMP7 and b-Catenin are neither plausible nor discussed.

Response: In the present study, mRNA and protein levels of MMP-7 were down-regulated in orthotopic tumors and hepatic metastatic tumors after 5-FU, WCA or 5-FU+WCA treatments, as detected using immunoblotting, IHC and real-time PCR. The orthotopic transplant model used in this study is a relatively natural nude mouse model of hepatic metastases, and the metastasis rate is not very high. Only 3 to 5 animals in each group developed liver metastases, which is not very powerful on a statistical point of view. In a future study, we will increase the sample size and this problem may be resolved. We discussed the discrepant results about MMP-7 expression as a limitation of this study.

6. What is the effect of WCA on colorectal cells in vitro? A simple proliferation/cytotox test using at least HCT116 must be provided or – if possible – cited from the literature.

Response: We thank the Reviewer for this input. Treatments with different concentrations of WCA for different durations have been performed in HCT-116 cells. The results have been added to the manuscript (Figure 1C, 1D, see below).



Minor points:

1. There are several smaller mistakes in style and form as for example Catenin versus catenin. Another example is the use of 0 in the 4th line of the discussion – "close to zero" would be superior. If finally accepted, the manuscript should thoroughly be revised concerning these things.

Response: The manuscript was thoroughly proofread by an English native speaker and little mistakes have been corrected.

2. Since the WCA alone did not significantly reduce hepatic metastasis, the title of the manuscript has to be changed!

Response: We changed the title to: 'Combination of Weichang'an with 5-fluorouracil Suppresses Colorectal Tumor in a Mouse Model'.

3. The treatment started 7 days after orthotopic tumor implantation – this is very early. At least, the authors should comment on that in the discussion. Would a later treatment still have effects on tumor development?

Response: We thank you for this comment. The animals had mostly recovered from the surgery 7 days after implantation. Tumor piece could be palpated 2 weeks after implantation and orthotopic tumor formation was confirmed by pathoanatomy. Considering that traditional Chinese medicine requires several days to achieve the necessary blood concentrations needed to exert an antitumor effect, we started the

treatment 7 days after implantation. Whether later treatments still have effects on tumor development requires further research.

4. Classically, the nuclear translocation is analyzed by immunohistochemistry. The authors should ideally provide such data or at the very least comment on why they did use W-Blot instead.

Response: Using immunohistochemistry (Figure 4) and western blot (Figure 5), β-catenin protein was showed to be decreased in all treatment groups compared with the controls, while the difference at protein level between the groups was not as evident as with mRNA. We presumed that translocation from the cytoplasm to the nucleus may be more significant than the change in protein amount. In order to analyze the exact amount of β-catenin protein in cytosolic and nuclear fractions, western blot is a better quantitative method.

5. 2 digits after the decimal point should always be used.

Response: This was corrected.

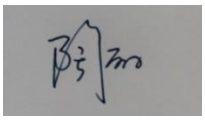
6. In the heading of Table 2 the authors write "HCT116 cell injection". Is this just a mistake – or is this a hint towards another technical procedure used in this study than the orthotopic implantation described?

Response: As described in the method section, the orthotopic implantation tumor model was established over two periods: subcutaneous tumor proliferation and cecum orthotopic implantation. Briefly, 2×10^7 HCT-116 cells were subcutaneously injected into the right axilla of nude mice. After 3 weeks, tumors were excised and cut into 1-2 mm^3 pieces using sterile techniques. Tumor sections were transplanted into the cecum of 48 nude mice by a purse-string suture.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology.*

Sincerely yours,



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