ANSWERING REVIEWERS

November 20, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 22551-revised.doc).

Title: Synergistic anticancer properties of DHA and 5-FU through interference in energy metabolism and cell cycle arrest in AGS cells

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Name of Journal: *World Journal of Gastroenterology*

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We appreciate the reviewers' comments and suggestions. All of the revisions we make to the revised manuscript are highlighted in the updated vision. The responses to reviewers' comments are as below.

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

1 Format has been updated.

Response: Format has been updated according to "Format for basic study" and the editor's advice.

2 Revision has been made according to the suggestions of the reviewer

Reviewer: # 02472104

Comments: Introduction: 1.The text: "This subsequently allowed lower dosages of 5-FU to be administered in combination with DHA in colorectal cancer[6, 7]. The results showed DHA supplementation had a powerful adjuvant activity and ….."is worded as if it refers to clinical trials and yet the studies quoted are from experimental models. Please re-word this.

Response: Thanks for your careful review! We have re-written the sentence as follow and highlighted in the revised manuscript. This subsequently allowed lower dosages of 5-FU to be administered in combination with DHA in the human colorectal cancer cell lines and colon adenocarinoma model. The studies in cancer cell lines and cancer-bearing animals showed DHA supplementation had a powerful adjuvant activity and has since emerged as an innovative approach to chemosensitize cancer cells.

2. The text: "The primary mechanisms by which DHA exert this apoptotic effect are believed to be the generation of reactive oxygen species and interaction with cellular signaling pathways though the detailed mechanisms remain to be investigated." is confusing. This needs to be re-worded.

Response: Thanks for your suggestions. We have revised this section as follow. Meta analyses examining an association between DHA consumption and the risk of gastric cancer are inconclusive, but high-dose DHA has been shown to induce apoptosis through activator protein-1(AP-1) activation in gastric cancer cells AGS. The studies further demonstrated that the mechanisms by which DHA in combination with 5-FU exert apoptotic effect are believed to be the regulation of apoptosis-associated gene expression in gastric cancer cells SGC7901 and MGC80. As a unique cellular organelle, mitochondria play a major part in apoptosis process and cellular energy metabolism. Thus the effect of co-administration of DHA with 5-FU on mitochondria of human gastric cancer cells need to be further investigated.

3. Last paragraph- use past tense (aimed not aims)

Response: we have revised the word in the manuscript

4. Methods: "Cell specimens "should be "cells"? Abbreviations should be defined such as PBST and mito-cyto.

Response: Thanks for your careful review. We have corrected the grammar errors in the manuscript. Abbreviations for PBST (phosphate-buffered saline with 0.1% tween 20) and Mito-Cyto (mitochondria cytosol) have also been defined in the revised manuscript.

5. Results: Are IC50 values precise to 0.01 ug/ml?

Response: Thanks for your review. Inhibition rate of cell growth was measured using the formula: inhibition rate (%) = [1-OD570 (experiment group)/OD570 (control group)] ×100. Absorbance values at 570 nm were measured by a microplate reader and are reported as a percentage of growth with respect to the controls. That is, inhibition rate was two decimal points. Based on the inhibition rate **(two decimal points)**, the values of IC50 for DHA or 5-FU was calculated by the median-effect equation **[reference 29 Chou TC. Preclinical versus clinical drug combination studies. Leuk Lymphoma 2008; 49: 2059-2080 in the revised manuscript]**.As a result, IC50 values for DHA or 5-FU was also two decimal points.

6. Discussion: The phrase: "agent 5-FU is the first-line chemical intervention" this needs to be worded in terms of "first-line chemotherapy". Instead of "more novel ", the term "new" would work better.

Response: Thanks for your suggestions. We have replaced the words as your suggestion.

7. It is difficult to follow the discussion when literature results are given in μM DHA and the present results are given in ug/ml DHA. Please put the corresponding μg/ml in parenthesis for literature results.

Response: Thanks for your careful review. The concentration of DHA in literature **[reference 17,27 and**

28 in the revised manuscript] are given in μM, such as markedly high concentrations of 100-150 μM **(32.85 - 49.28 μg /mL)** or 180-200μM **(59.13 - 65.70 μg / mL)**, respectively. We have calculated the corresponding μg/ml in parenthesis in the revised manuscript.

8. "to 12.9μg/mL, 34.17μg/mL" should be "to 12.9 μg/mL and 34.17μg/mL". Again, it is doubtful that the assays are precise to two decimal points, and be consistent in using the same number of decimal points. Same applies for "be 30 μg/mL, 12.5 μg/mL".

Response: Thanks for your careful review. 12.9 and 34.17 are IC50 values for 5-FU and DHA administered in combination for 24h, respectively. This review is the same as "Are IC50 values precise to 0.01 ug/ml?" Consequently, our response please sees response to 5. Results: Are IC50 values precise to 0.01 ug/ml? In addition, we have used the two decimal points to 30.00 μg/mL, 12.50 μg/mL in the revised manuscipt.

9. Decimal point issues also exist in Figure 1 legend. Are the DHA and 5-FU concentrations used in this work something that could be achieved *in vivo*?

Response: For typical *in vitro* drug combination studies, it is necessary to approximate the IC50 values for each compound to estimate a working dose-range **[reference 29 Chou TC. Preclinical versus clinical drug combination studies. Leuk Lymphoma 2008; 49: 2059-2080 in the revised manuscript]**. Therefore, in the current study a series of concentrations of 5-FU (1.5625, 3.125, 6.25, 12.5, 25, 50 μg /mL) and DHA (7.5, 15, 22.5, 30, 37.5, 45 μg /mL) were first tested to determine their individual IC50 values against AGS cells. Based on the IC50 values obtained for DHA and 5-FU from the MTT assay, the optimal treatment concentrations for DHA and 5-FU were determined to be 30.00 μg /mL, 12.50 μg /mL, respectively. The optimal concentration of DHA and 5-FU in this work was just determined for study *in vitro.* As far as the treatment concentrations for DHA and 5-FU *in vivo* were concerned, studies should be further performed.

10. This decrease was also found to be synergistic in a statistically significant manner." A decrease cannot be synergistic but the combination of two compounds can be.

Response: Thanks for your suggestions. Yes，just the combination of two compounds can be synergistic in a statistically significant manner. We have re-worded this sentence as follow. The combination of two compounds was also found to be synergistic in a statistically significant manner.

11. The paragraphs in the discussion need to be tied together better instead of jumping from topic to topic without any pre-amble.

Response: Thanks for your careful review. In our study, the evaluation of synergistic anticancer

properties of DHA and 5-FU focused on cell cycle arrest and energy metabolism in AGS cells. Consequently, we have also planed the discussion in the same order.

12. Is there any mechanistic connection between the cell cycle effects and the mitochondrial toxicity observed? "a marked reduce" should be "a marked reduction"

Response: Based on results in this study, we could not directly draw a conclusion that there is mechanistic connection between the cell cycle effects and the mitochondrial toxicity. Theoretically, the mitochondrial toxicity could result in energy deficiency in cancer cells, which would influence the cell cycle progression, because the cell cycle progression needs the energy. In addition, we have replaced the word "reduce" with reduction.

13. Is a decrease in mitochondrial activity a good thing in light of the fact that cancer cells display the Warburg effect? This is confusing on page 11. It would be useful to comment on the cell line used and its relevance to human gastric cancer.

Response: Yes, a decrease in mitochondrial activity is a good thing for inhibition of cancer cell growth. Normal cells depend principally on mitochondrial oxidative phosphorylation to produce ATP for their metabolic activities. In 1920s, Warburg suggested cancer cells rely on chiefly glycolysis as the major source of ATP **[reference20 in the revised manuscript]**. However, at present many researchers have showed that mitochondria have distinct functions in most cancer cells and are still the primary contributors to ATP production **[reference21-24 in the revised manuscript]**.Our study showed that the expression of complex I, II and V in mitochondria was markedly reduced in AGS cells following treatment with DHA and 5-FU alone or in combination. In other words, the two test compounds could inhibit mitochondrial oxidative phosphorylation and cellular energy metabolism in AGS cells. Consequently, a decrease in mitochondrial activity is a good thing for inhibition of cancer cell growth. In addition, the results obtained in human gastric cancer cells AGS could just provide the clue to the chemotherapy of human gastric cancer in clinical practice.

14. Table 1: Does a CI less than 1 indicate negative synergism? In methods, can the difference between negative and positive synergism be defined to facilitate interpretation of the results?

Response: Thanks for your careful review. A CI less than 1 indicate positive synergism, not negative synergism. The combination index (CI) were calculated using the formula CI=%AB/%A×%B, where %A and %B are the inhibition rate of DHA and 5-FU alone on AGS cells growth, and %AB is the inhibition rate of DHA and 5-FU in combination on AGS cells growth **[reference 25 in the revised manuscript]**.When CI = 1,

the effect between two compounds is considered additive; when CI is significantly greater than or less then 1, the effect is considered negative or positive synergism, respectively**[reference 25,29 in the revised manuscript]**.

15. Figure 1: It is not given which compounds were used to treat the cells in panels A-C. In panel D, it appears that there is an additive effect after 48 hours (not synergistic) and no benefit of the combination at 24 hours. How do the results support synergism?

Response: Thanks for your valuable suggestion. In Figure 1, we have clearly shown which compounds were used to treat the cells in the revised manuscript. In Table 1, CI for the interaction between the inhibitory effect of DHA in combination with 5-FU on cell growth for 48hours was 0.057±0.013. It was less than 1, (P<0.05 compared to the additive combination index of 1 by Student's t-test), so the interaction between the inhibitory effect of DHA in combination with 5-FU was positive synergism.

16. Please remove "could" from "could notably increased" and from "could markedly suppressed". The text in the Figure 1 legend is too detailed, making it hard to read.

Response: Thanks for your careful review. We have removed the word "could" in the in the revised manuscript. Meanwhile, we have also shortened the Figure 1 legend.

3 References and typesetting were corrected

Thank you again for your much effort on our manuscript.

Sincerely yours,

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