December 25, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 6634-review.doc).

Title: Naofen promotes TNF-α-mediated apoptosis of hepatocytes by activating caspase-3 in lipopolysaccharide-treated rats**.**

Author: Jun-Hua Fan, Guo-Gang Feng, Lei Huang, Guo-Duo Tang, Hai-Xing Jiang, Jing Xu.

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

1 Format has been updated

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

(1) Dear 00462396 reviewer, thank you very much for reviewing our paper.

Question 1: More elaborated insight of parameters/cytokines/… that contribute to the induction of apoptosis induced by lipopolysaccharide must be provided.

Answer: In order to identify the nature of these unknown mediator(s), we examined the effects of the combination of TNF-α with either IL-1, IL-6 and interferon-γ (10 ng/ml for each) or inhibitors of NF-κB, such as BAY 11-7082 and DHMEQ on naofen expression, but either combination with TNF-α or metabolites of TNF-α treated with trypsin, failed to enhance naofen expression in primary hepatocytes. (at line16, 17, 18, 19, 20 on page 12).

Question 2: This paper assumes that apoptosis is the only mechanism of cell death. By only considering the latter, one can not exclude the role of other modes of cell death, in particular necrosis, which could also have an impact in the setting addressed by the authors.

Answer: This is follow-up study of previous published research. Under septic conditions,

LPS-induced hepatocyte death may have a role in liver dysfunction, possibly associated with the apoptosis of hepatocytes. LPS does not directly have pathogenetic roles, but rather the effects are mainly dependent on the production and release of potent inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-10 and so on. Naofen was increased in hepatocytes, causing apoptosis in LPS-treated rats liver. Hence, the present study was undertaken to examine whether naofen participates in the TNF-α-mediated apoptosis of hepatocytes in LPS treated rat.

Question 3: the number of biological and technical repeats, and independent experiments, the number of rats per group, … are lacking.

Answer: Duplicate cultures were prepared for each treatment, and independent experiments were performed at least four times (at line 8, 9 on page 6). Ten rats were used for each time point (at line 18 on page 5).

(2) Dear 00058872 reviewer, thank you for recommend, further comment on the role of the spleen in the liver diseases will be investigated in future experiment.

(3) Dear 02638641 reviewer, thank you very much for reviewing our paper

Question 1: As it was described in the manuscript that treatment with TNF-α alone did not cause the apoptosis of primary hepatocytes, the conclusion of the manuscript should be carefully reconsidered.

Answer: In conclusion, naofen may be involved in part in LPS-induced hepatocyte apoptosis, which is mediated by mediators including TNF-α released from KCs. Naofen elicits inhibitory action on the expression of Bcl-2 and Bcl-xL, releasing cytochrome *c* from mitochondria, and activating caspase-3, finally leading to the apoptosis of hepatocytes... (at line4, 5, 6, 7 on page 13).

Question 2: If KCs treated with LPS stimulated hepatocellular apoptosis via TNF-naofen pathway, the inhibitors to block naofen expression should be used in the study to confirm the conclusion.

Answer: The increased naofen expression in LPS-treated rat as well as the effects of KC-CM on naofen expression in hepatocytes was clearly blocked by pre-treatment with anti-TNF-α antibody (Figure 1, Figure 4). Moreover, naofen siRNA inhibited the increase in naofen protein induced by 6 h KC-CM, and naofen-siRNA also prevented KC-CM-induced caspase-3 activation in previous study… (At line 22, 23 on page 10).

Question 3: In the introduction part, more background information about naofen should be mentioned to emphasize the aim or rationale of the study.

Answer: Naofen is a member of aspartate-tryptophan (WD) 40-repeat protein family and acts as an intracellular protein reactive to anti-verotoxin II antibody[16]. In deoxycorticosterone-induced renal hypertension of rats, Naofen is increased in vascular endothelial cells and suppresses nitric oxide synthesis[16]. Naofen also induces apoptosis in streptozotocin-induced diabetic rat kidney[17] and mediates spontaneous and TNF-α induced apoptosis in human embryonic kidney (HEK) 293 cells[18]. Most importantly, naofen is increased in hepatocytes, causing apoptosis in LPS-treated rat liver[19]. (at line19, 20, 21, 22, 23, 24 on page 4).

Question 4: How many times were repeated in the vitro experiments using primary hepatocytes or KCs. It should be mentioned in the manuscript.

Answer: Duplicate cultures were prepared for each treatment, and independent experiments were performed at least four times (at line 8, 9 on page 6).

Question 5: Had the TUNEL staining and Western blotting been quantitatively analyzed?

Answer: Changes in target protein levels were measured quantitatively using Image J (Free software made by NIH initiative) (at line 13, 14, 15 on page 7). For each sample, five high-power fields (×200) were randomly selected, each containing an average of 400 cells, and the number of apoptotic cells was counted for each field. Apoptosis index (AI) (%) = number of positive cells/number of total cells x 100% (at line 1,2, 3, 4 on page 8).

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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