January 10, 2013

*World Journal of Gastroenterology*

Editorial Office

Dear Editors:

Please find enclosed the edited manuscript in Word format (file name: 1219-revision.docx).

**Title:** Influences of Kupffer cells and platelets on ischemia-reperfusion injury in mild steatotic liver

**Author:** Koichi Ogawa, Tadashi Kondo, Takafumi Tamura, Hideki Matsumura, Kiyoshi Fukunaga, Tatsuya Oda, Nobuhiro Ohkohchi

**Name of Journal:** *World Journal of Gastroenterology*

**ESPS Manuscript NO:** 1219

We are very grateful for the reviewers’ detailed comments on our manuscript. The comments have been helpful in allowing us to revise and improve our manuscript. Below, we have attempted to answer the comments and questions raised by the reviewers.

First, we changed our manuscript’s title to “**Influences of Kupffer cells and platelets on ischemia-reperfusion injury in mild steatotic liver**” to reduce the number of words. In addition, we made the abstract more informative and structured.

**Response to Reviewer #1 (No. 00038529)**

1. Abstract

Please give the full name for a specialized term, instead of abbreviation alone, when it appears for the first time in the abstract.

Following the reviewer’s comment, we changed first ‘ALT’ to ‘alanine aminotransferase’ in the abstract.

2. Introduction

The author explored the hepatic injury in mild steatotic after ischemia and reperfusion in the text, it is better to give more information concerning the necessity of this study. What is the incidence of mild steatotic in clinic? What is the clinical significance of this study?

According to the reviewer’s comment, we added the following sentences to the second paragraph of the Introduction in the revised manuscript (page 5, from line 12 to 15 and from line 17 to 21).

Thrty percent of the population in Japan and Western countries suffers from steatotic liver, and the percentage is still increasing [1, 2]. Most of these patients have been diagnosed by abdominal ultrasonography screening, even though elevation of serum liver enzyme levels was detected in a lesser percentage [1]. Fishbein et al. reported that liver enzyme levels were not elevated in the steatotic liver in which the proportion of hepatocytes with fat deposition was less than 18% [3]. Steatotic liver even in the mild degree is a risk factor for complication after liver resection [4].

[1] Federico A, et al. *World J Gastroenterol* 2010; **16**: 4762-4772.

[2] Omagari K, et al. *J Clin Biochem Nutr* 2009; **45**: 56-67.

[3] Fishbein MH, et al. *J Pediatr Gastroenterol Nutr* 2003; **36**: 54-61.

[4]de Meijer VE, et al. *Br J Surg* 2010; **97**: 1331-1339.

3. Materials and Methods

1) In the third part (“Surgical Procedure”), the author said that blood samples were taken for analysis of enzyme activity at the same time as IVM. Please clarify which enzyme was detected, and how to collect the blood? Whether blood collection influences liver microcirculation? If it does, how to avoid?

Answer to reviewer

We evaluated alanine aminotransferase (ALT) as one liver enzyme. We collected the blood samples from a catheter placed in the left carotid artery. At 120 min of reperfusion, total body blood was taken for euthanasia. We added these descriptions in the ‘Surgical Procedure’ part of Materials and Methods (page 7, from line 8 to 10).

According to the textbook, an adult rat has a blood volume of approximately 70ml/kg, and it is possible to withdraw up to 10% of this volume at any one time without causing significant effects [1]. Blood volume of rats of 250-300 g is 17.5-21 ml, and the amount of one blood collection is 0.5ml (less than 3% of blood volume). Through the evaluation of the velocity of post-sinusoidal venules, it was confirmed that microcirculation was maintained before and after the collection of blood sample.

[1] Waynforth HB, Flecknell PA. Experimental and surgical technique in the rat. 2nd ed. San Diego: ACADEMIC PRESS INC, 1992; 68-69.

2) In the fourth part (“Platelet Preparation”), the author said that platelets were labeled by rhodamine-6G in a concentration of 50 mL/ml whole blood. The concentration seems too high. Please check it.

Following the reviewer’s comment, we corrected ‘50 mL/ml’ to ’50 μl/ml’ in the ‘Platelet Preparation’ part of Materials and Methods (page7, line 16).

3. Results

1) In the third part of the results, the author only give the number of KC in acini before ischemia. However, in the related part in the Materials and Methods, the author said that liver tissue was only taken after reperfusion (at the end of experiment). Please check and add the result of the number of KC in acini after reperfusion.

As the reviewer commented, we investigated immunohistochemically the number of KCs before ischemia and 120 min after reperfusion. We added the following contents to the top of ‘***Immunohistochemical Study of KCs***’ part of Materials and Methods (page 9, from line 1 to line 5).

We immunohistochemically assessed the number of KCs in the acini. To compare the differences between the normal liver group and the mild steatotic liver group before ischemia and after reperfusion, liver tissues were obtained from each group both before ischemia and at the end of surgical procedure, and from another animal before ischemia.

In addition, we added the results of the number of KCs in acini 120 min after reperfusion to the end of ‘***Number of KCs in Acini***’ part of Results (page 11, from line 26 to line 27) and Figure 4B. The number of KCs in acini was lower in the mild stetatotic liver group than the normal liver group even after 120 min of reperfusion.

2) IL-6 is a marker of KC number and activity. However, IL-6 didn’t decrease, although a tendency occurred, after I/R in mild steatotic compared with normal. The author should repeat this experiment due to the big standard deviation.

Following the reviewer’s comment, we performed the re-examination of serum IL-6 and changed Figure 6B according to the results. IL-6 levels tended to be lower in the mild steatotic liver group compared with in the normal liver group, however, there was no significant difference between them (after 120 min of reperfusion, *P* = 0.09). Therefore we changed the sentence in the ‘***Serum ALT and IL-6 Levels’*** part of Results (page 12, line 11).

4. Discussion

1) The author should highlight the main innovative points and clinical significance of this research.

The main innovative points of the present study are that the IR injury in mild setatotic liver was attenuated compared with normal liver, and that we demonstrated those were results of the reduction of the interaction between KCs and platelets due to the decreased number of KCs. We added these descriptions in the 1st paragraph of discussion (page 13, from line 10 to line 13).

IR injury is involved closely with complications after hepatic resection in steatotic liver [1]. It is known that steatotic liver is a risk factor for postoperative complications [1, 2]. On the other hand, there is a report that postoperative liver failure was slight in mild steatotic liver, so mild steatotic liver would be indication of hepatic surgery [3]. Moreover, mild to moderate seteatotic livers were accepted as a marginal graft in transplantation [4, 5]. Our study suggested that IR injury does not depend on the degree of fat deposition. In addition, our results provided some evidence that the postoperative outcomes after liver resection or transplantation are not aggravated in mild steatotic liver. We added these sentences in the 5th and 6th paragraphs of discussion (page 15, from line 13 to line 15 and from page 10, line 28 to page 16, line 1).

[1] McCormack L, et al. *Ann Surg* 2007; **245**: 923-930.

[2]de Meijer VE, et al. *Br J Surg* 2010; **97**: 1331-1339.

[3] Cho JY, et al. *Surgery* 2006; **139**: 508-515.

[4] Perez-Daga JA, et al. *Transplant Proc* 2006; **38**: 2468-2470.

[5] Briceno J, et al. *Transpl Int* 2005; **18**: 577-583.

2) What is the possible mechanisms for the decrease in KC number and KC-platelet interaction? The decrease in KC-platelet interaction is due to the decrease of KC number or the decrease of platelet adhesion ability? The author should give more information in discussion.

Answer to reviewer

As described in the discussion, in the present study, we supposed that change in a nutrient condition induced by CDD might lead to decrease of KCs as well as mild steatotic liver. Guo et al. reported similar results of decreased KCs in a different steatotic liver model induced by palmitoleate [1]. The physiologic mechanism of the influence that steatotic liver has on the number of KCs is not reported, and it is difficult to clarify it under the present conditions. It will be necessary to evaluate the change in the number of KCs according to the enhancement of the degree of steatosis or the change in the number of KCs in the mild steatotic liver induced by a method other than CDD in future.

The number of adherent platelets in sinusoids increased along with the reperfusion time both in the normal liver group and in the mild steatotic liver group. This suggests that the adherent ability of platelet was not reduced in the mild steatotic liver group. Therefore, we considered that the reduction in KC-platelet interaction was result of the decreased number of KCs in the mild steatotic liver. We added these sentences in the 2nd paragraph of discussion (page 14, from line 6 to line 11).

[1]Guo X, et al. *PLoS One* 2012; **7**: e39286*.*

3) How to explain the discriminative manifestation between mild and severe steatotic liver after I/R injury?

Hepatic resection is associated with IR injury, which is a potential factor responsible for postoperative complication including liver failure in steatotic patients [1]. The decreased tolerance of steatotic liver to IR injury is a result of impaired microcirculation due to hepatocytes with fat deposition [2]. Between mild and severe steatotic liver, there are differences in microcirculation disturbances due to differences in the degree of fat deposition. Recent meta-analysis revealed a significant association between degree of steatosis and increased risk of postoperative complications and mortality [3]. Several investigators reported that postoperative complications, especially infectious complication, and mortality increased in patients with severe steatotic liver compared with in those with mild setatotic liver [3, 4]. It will be necessary to evaluate IR injury in the moderate to severe steatotic liver with a similar experimental model in the future. We added these sentences in the 5th paragraph of discussion (page 15, from line 18 to line 27).

[1] McCormack L, et al. *Ann Surg* 2007; **245**: 923-930.

[2] El-Badry AM, et al. *Hepatology* 2007; **45**: 855-863.

[3]de Meijer VE, et al. *Br J Surg* 2010; **97**: 1331-1339.

[4]Kooby DA, et al. *J Gastrointest Surg* 2003; **7**: 1034-1044.

5. Reference

The references cited are too many. Please reduce the number of references.

According to the indication from the reviewer, we reduced the number of references to 39.

6. Figures

The annotations of abscissa in Figure 3A, 3C, 4A, 6A and 6B are not proper. “Time after reperfusion” may be better.

According to the suggestion from the reviewer, we corrected the annotations of abscissa in Figures.

7. There are some mistakes in grammar and spelling, which should be revised.

As the reviewer’s comment, the revised manuscript was checked again by a native speaker of English for English corrections including grammar and spelling.

8. A space between digit and unit is needed, which is missing throughout the text. Please add it.

According the reviewer’s indication, we revised our manuscript.

9. Please unify the expression for a unit, e.g., “minutes or min”.

As the reviewer’s comment, we unified the expression for a unit, i.e., ‘s’, ‘min’, and ‘h’.

**Response to Reviewer *#2 (00038428)***

The authors might explain better the effects of IR injury and of the time reperfusion, authors should better explain damage from reperfusion and in particular should better explain the choice of the 120 min of reperfusion.

As described in the Discussion, hepatic IR injury was divided into two distinct periods. The early period of IR injury is characterized by activation of KCs, which generate reactive oxygen species and aggravate the injury occurring up to 120 min after reperfusion. In addition, in response to the exposure to activated KCs, neutrophils accumulate in the post-ischemic liver. In the late period of IR injury, inflammatory responses from accumulating neutrophils induce hepatocyte injury, which appears more than 6 h after reperfusion.

Elevated liver enzymes and apoptosis of hepatocytes and sinusoidal endothelial cells can already be observed already in the early period [1, 2]. Reduction of the early period of IR injury, for instance KCs depletion, leads also to inhibition of injury in the late period [3]. Most of the events that determine the extent of IR injury, such as KC activation, platelet adhesion to KCs, and neutrophil accumulation, have occurred in the early period of IR injury. Therefore, we have focused on observation until 120 min after reperfusion. We added these sentences in the 2nd paragraph of the Discussion (page 13, from line 17 to line 24 and page 14, line 1 to line 4).

[1]Tamura T, et al. *J Surg Res* 2012; **178**: 443-451.

[2]Sindram D, et al. *Gastroenterology* 2000; **118**: 183-191.

[3] Giakoustidis DE, et al. *Hepatogastroenterology* 2003; **50**: 1587-1592.

The manuscript has some typographical errors:

-“Experimental design" delete space after n = 6;

As the reviewer indicated, we checked and revised.

-“References” n° 3 Fat. A Matter; n°5 delete []

As the reviewer indicated, we checked and revised.

-“Discussion” add reference of Shono et al (n°36);

As the reviewer commented, we added the reference in the appropriate part (page 14, line 29). Reference number was changed to ‘33’.

-Figure 3A: add the significative (\*)

As the reviewer indicated, we added the significative in Figure 3A.

We have thoroughly revised the manuscript on the basis of comments provided by the reviewers, and the manuscript was checked by a native speaker of English. We thank the reviewers for their careful reading of our manuscript and for their constructive criticism. We would be grateful if this manuscript could be re-reviewed to assess its suitability for publication in the *World Journal of Gastroenterology*.

Thank you for your consideration.

Sincerely yours,

Koichi Ogawa, M.D.

Corresponding author: Nobuhiro Ohkohchi, M.D., Ph.D.

Department of Surgery, Doctoral Program in Clinical Science, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan

Tel: +81-29-853-3221, Fax: +81-29-853-3222

 E-mail: s1130425@u.tsukuba.ac.jp, nokochi3@md.tsukuba.ac.jp.