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Response to Reviewer A

Major comments

Comment 1: The ostensible reason for the study was to assess the safety of administration of iron to patients with cirrhosis but in clinical practice, one would only contemplate administering iron to patients who have evidence of iron deficiency. Based on the data provided in Table 1, there is no evidence that the anemia in the cirrhotic rats was the result of iron deficiency (i.e., serum iron, transferrin saturation, ferritin were all the same or higher in LC group vs controls). Microcytosis (as discussed on page 12) is insufficient to make a diagnosis of iron deficiency in the face of these other data indicating lack of iron deficiency.) This does not invalidate the findings of the study but it needs to be noted that this model is not one of iron deficiency.

Reply 1: I appreciate your constructive comments.

It is common that high frequency of hematological abnormalities is followed by advanced liver disease. Iron deficiency anemia is frequent among complications of chronic liver disease. Since the liver plays a major role in iron homeostasis, it is clear that liver disease with various etiology are directly related to alteration of iron homeostasis, which makes the diagnosis of iron deficiency anemia very difficult. The superiority of ferritin over other parameters to diagnose iron deficiency has been proven by several studies, so ferritin level is commonly used. However, the fact that ferritin is

an acute phase protein, affected not only by the iron status of the body, but also by acute or chronic inflammation, malignancies and liver disease, makes the interpretation of test results challenging. According to the recent study, in the general population, a cut off value of ferritin to diagnose iron deficiency is < 12 μ/L. However, when it comes to patients with inflammation of chronic liver disease, the level is significantly higher with the best predictor being < 100 μ/L (or higher) [1, 2]. In systemic analysis, it was found that measurement of serum ferritin was particularly helpful, even in a population with chronic liver disease or inflammation, but the test results in these patients needs to be interpreted with a different cut off value as mentioned above [3]. According to above described evidence, elevated ferritin level of our study in LC induced rats comparted to control group is explained by the inflammatory state by liver disease. Accordingly, the cut-off value of ferritin also should be interpreted based on disease state of rats, which shows that rats in control group are not in the iron deficiency (ferritin > 12 μ/L) and rats in LC groups are in the iron deficiency (ferritin < 100 μ/L). Thus, treatment of iron deficiency can be useful in rats with liver cirrhosis, even the ferritin level was higher than the limit range of healthy population.[4]

Moreover, recently, numerous evidence support the importance of treating functional iron deficiency.[5-7] In inflammatory states by liver disease, although serum levels of ferritin are high, iron is unable to circulate or be delivered to the bone marrow, which in turn leads to iron-restricted erythropoiesis.[2, 8] Previous study reported by Rossler et al. have made the evidence highlighting that preoperative iron deficiency, even in the absence of anemi,a is an important prognostic marker for postoperative

mortality.[6]

Because to test safety of iron administration was our primary outcome, we would not expect iron administration to have a major impact on the anemia without iron deficiency rat model. However, recent evidence of importance of absolute and functional iron deficiency in both anemic and non-anemic patients may validate the findings of the study.

Changes in the text: "However, we would not expect iron administration to have a major impact on the anemia since this is not a model that causes iron deficiency. In inflammatory states by liver disease, although serum levels of ferritin are high, iron is unable to circulate or be delivered to the bone marrow, which in turn leads to iron-restricted erythropoiesis (30,31). Previous study have made the evidence highlighting that preoperative iron deficiency, even in the absence of anemi,a is an important prognostic marker for postoperative mortality (32). Recent evidence of importance of absolute and functional iron deficiency in both anemic and non-anemic patients may validate the findings of the study." (see Page 17, line 13-20)

Comment 2: The authors imply that AST and ALT levels are indicative of cirrhosis (line 1, page 7). This is completely incorrect—the aminotransferases are markers of hepatocellular injury but are not predictive of the degree of fibrosis. This statement needs to be deleted. Likewise, the data regarding AST and ALT levels does NOT belong in the first paragraph under the Results on page 10 ("Confirmation of liver fibrosis"). This section and the section "Liver fibrosis and iron deposition" should be combined

into a single paragraph.

Reply 2: We completely agree with your comment. Although we confirm the liver fibrosis by gross histopathological examination and checked AST and ALT was measured just for checking the state of hepatocellular injury, our statement can make confusion for readers. Thus, we deleted this statement and revised the sentence as follows.

We also revised result section related to liver function test, as you mentioned.

Changes in the text: "Blood samples were obtained at 6–10 weeks, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured to check the state of hepatocellular injury." (see Page 7, line 5-7)

"**Main experiment: Liver fibrosis and iron deposition**

Histological examination indicated that after CCL4 administration, there were significantly greater degrees of fibrosis in the LC and LC-iron groups than in the control group (Figure 2A). The level of iron accumulation was significantly greater in the LC-iron group than in the LC group at all measurement points (Figure 2B). The degrees of fibrosis were comparable between the LC-iron and LC groups on days 2, 14, and 28 ($P=1.0$, $P=0.25$, and $P=1.0$, respectively). The degree of hepatic iron accumulation was significantly greater in the LC-iron group than in the LC group on days 2 ($P<0.001$), 14 ($P=0.029$), and 28 ($P<0.01$). Histopathological examination indicated ballooning cells, fatty changes, fibrosis, and iron accumulation in liver tissue (Figure 2). Focal iron accumulation was observed in the portal triad in the LC group, but diffuse accumulation was present in the hepatic triad and liver lobules in the LC-iron group. Iron deposition

(visualized using Prussian blue staining) was mainly deposited to reticuloendothelial cells in the LC-iron group but showed mixed distribution in non-reticuloendothelial hepatocytes and reticuloendothelial cells in LC group." (see Page 10, line 7 - 21)

"***Liver function test***

During the test period, AST and ALT levels were significantly higher in both the LC and LC-iron groups than in the control group ($P<0.01$). Mean AST and ALT levels of Control group throughout the main experiment were 98.4 ± 14.9 IU/L and 75.9 ± 12.7 IU/L, respectively. It was negligible to be showed in the graph to put together with LC and LC-iron group so we did not show the control group in the figure 4. AST/ALT levels were not significantly different between the LC and LC-iron groups at 0, 2, 14, or 28 days (Figure 4). ALT levels on days 4 and 7 ($P<0.03$ and $P<0.001$) and AST level on day 7 ($P<0.001$) after treatment, were significantly lower in the LC-iron group than in the LC group (Figure 4)" (see Page 12, line 4-13)

Comment 3: Several details of the experimental design are confusing or unclear. Specifically, the timeline of the iron administration relative to induction of cirrhosis needs to be clarified. Perhaps a **diagram** could be provided. Secondly, on page 7, line 4-5, states that liver fibrosis was confirmed by histopathological examination of "2 rats per week." Which weeks??? Third, lines 9-10 on the same page state that one of the authors was aware of the group allocations, while lines 14-15 state that the authors were blinded to the animal treatments. If the former is true, the latter is not.

Reply 3: Thank you for your constructive comment. As you suggested, we added a

diagram including the timeline of the iron administration relative to induction of cirrhosis and autopsy for pathologic exam.

For the third comment, we described contents related to “Blinding”.

Changes in the text: Please check the Diagram added as Figure 1.

“The investigators, except the principal investigator, were blinded to the animal treatments.” (see Page 7, line 22-23)

Comment 4: Similarly, the timing of the blood samples (“before injection;” also some samples were apparently obtained at euthanasia as well) as described on page 8 is not clear.

Reply 4: As you suggested, we added the timing of the blood samples in a diagram (Figure 1).

Changes in the text: Please check the Diagram added as Figure 1.

Comment 5: The description of the histopathological examination/quantification of fibrosis is inadequate. The authors state that hematoxylin and eosin was used to identify fibrosis. H&E is used to assess morphology; it is not a specific stain for connective tissue or fibrosis, for which a trichrome state is most often used. Elsewhere in the manuscript, the authors state that they used Sirius Red. This stain is used for quantitative morphometry/image analysis. It is not stated in the paragraph on page 9 whether image analysis was done for fibrosis quantitation; this should be clarified. It is also unclear what was “immunolocalized,” as none of the techniques described

involved an immunologic technique—Sirius Red and Prussian Blue are histochemical methods. Finally, the histopathological scoring system used here (one for chronic hepatitis) is not appropriate for this model, as the early injury in CCl4 does not involve the portal tracts. It would be helpful to enlist the assistance of someone with expertise in hepatopathology to review these data.

Reply 5: We appreciate your constructive comments. (1) We agree with your comment that H&E is not a specific stain for connective tissue or fibrosis. Therefore, as you mentioned, for quantitative morphometry/image analysis of the liver fibrosis, we decided to conduct additional experiment using Sirius red staining for all tissue samples because sirius red staining was used for limited number of tissue samples. However, it took time to perform experiment of all samples. Thus, if you give more time to conduct additional experiment for stored tissue samples, we can complete the experiments and provide more specific analysis results about liver fibrosis with appropriate scoring system under assistance of hepatopathology expertise.

We are sorry for wrong description. As you mentioned, we revised "immunolocalized" to "Histochemical localization".

Changes in the text: 'Histology and quantification of fibrosis' in Method section and 'Main experiment: Liver fibrosis and iron deposition' in rsult section will be revised after obtaining the rusults of the additional experiment.

"Histochemical localization in the liver tissue of each rat was calculated using a computer and image analyzer and expressed as the percentage of the area per millimeter of positive iron deposit staining." (see Page 9, line 15-17)

Comment 6: It is not necessary to include the long, confusing string of aminotransferase levels +/- variances in the first paragraph on page 11. Highlight the significant findings and refer back to the figure.

Reply 6: We agree with your opinion that the first paragraph on page 11 is confusing. As you commented, we revised manuscript with highlighting the significant findings as follows.

Changes in the text: "Liver function test

During the test period, AST and ALT levels were significantly higher in both the LC and LC-iron groups than in the control group ($P<0.01$). Mean AST and ALT levels of Control group throughout the main experiment were 98.4 ± 14.9 IU/L and 75.9 ± 12.7 IU/L, respectively. It was negligible to be showed in the graph to put together with LC and LC-iron group so we did not show the control group in the figure 4. AST/ALT levels were not significantly different between the LC and LC-iron groups at 0, 2, 14, or 28 days (Figure 4). ALT levels on days 4 and 7 ($P<0.03$ and $P<0.001$) and AST level on day 7 ($P<0.001$) after treatment, were significantly lower in the LC-iron group than in the LC group (Figure 4)." (see Page 11, line 4-13)

Comment 7: The cellular localization of iron deposition (hepatocytes vs Kupffer cells vs mixed) should be described in the section on iron deposition (page 11).

Reply 7: Following the comment, we describe the cellular localization of iron deposition in the section on iron deposition.

Changes in the text: "Iron deposition (visualized using Prussian blue staining) was mainly deposited to reticuloendothelial cells in the LC-iron group but showed mixed distribution in non-reticuloendothelial hepatocytes and reticuloendothelial cells in LC group." (see Page 11, line 19-21)

Comment 8: Page 16, from lines 3-14 onward: The authors provide a highly speculative argument regarding ALT levels and hepcidin. This needs to be described as such, as there are no data showing this relationship in the liver.

Reply 8: Thank you for your comment. As you commented, we changed manuscript as follows.

Changes in the text: "Second, an elevated hepcidin level following iron injection may be related to reductions in AST and ALT levels. In our analysis, the hepcidin level was significantly greater in the LC-iron group than in the LC group, which can be explained by natural reaction following high dose of iron injection. Some of previous researches have described that an acute elevation of hepcidin can reduce inflammatory cytokine levels, thereby lowering the ferritin level. Urrutia et al. demonstrated that hepcidin pretreatment has the potential to relieve the damage caused by inflammatory signaling (27), via downregulation of IL-6 and TNF-α expression in astrocytes and microglia. This intracellular mechanism can thus protect neurons from oxidative stress. In addition, treatment with ad-hepcidin under non-inflammatory conditions can protect neurons from iron loading (27,28). There were significantly lower degrees of inflammation and oxidative stress in liver tissues in the LC-iron group than in the LC group and the

significant reductions in ALT and AST levels might be attributed to concomitant decreases in the levels of inflammatory and oxidative stress markers. However, this is a speculative assumption since there are no data showing this relationship in the present study." (see Page 16, line 12 - Page 17, line 1)

Comment 9: The authors state that the effects of IV iron on anemia were not evaluated in their study (page 17, line 3). It's unclear why they feel the need to say this, since they did, in fact, measure hemoglobin levels in this study after iron administration. It would probably be more relevant to note that since this is not a model that causes iron deficiency, one would not expect iron administration to have a major impact on the anemia.

Reply 9: We appreciate your comment. Following your comment, we changed the manuscript regarding that one would not expect iron administration to have a major impact on the anemia since this is not a model that causes iron deficiency.

Changes in the text: "However, we would not expect iron administration to have a major impact on the anemia since this is not a model that causes iron deficiency. In inflammatory states by liver disease, although serum levels of ferritin are high, iron is unable to circulate or be delivered to the bone marrow, which in turn leads to iron-restricted erythropoiesis (30,31). Previous study have made the evidence highlighting that preoperative iron deficiency, even in the absence of anemi,a is an important prognostic marker for postoperative mortality (32). Recent evidence of importance of absolute and functional iron deficiency in both anemic and non-anemic patients may

validate the findings of the study." (see Page 17, line 13-20)

Comment 10: The images of the histopathology should be provided at a higher resolution and higher magnification in order to be interpretable.

Reply 10: As you commented, we provided new version of figures with a higher resolution and higher magnification.

Changes in the text: Please see newly added figure 2.

Minor comments

Comment 11: Page 12, line 20: P=0.458 is not significant.

Reply 11: I apologize for numeric error on this. We changed P-value as follows.

Changes in the text: "The serum ferritin level was significantly lower (P=0.045), but the C-reactive protein level higher (P=0.076), in the LC-iron group than in the LC group on days 2 and 14 (*Table 1*)." (see Page 13, line 4)

Comment 12: Page 14, lines 13-14: "hepcidin synthesis is altered"—it is not known whether hepcidin synthesis is altered, as most studies have examined only hepcidin gene expression

Reply 12: Thank you for your constructive comment. We change the term "synthesis" to "gene expression" as follows.

Changes in the text: "In chronic liver disease, Hepcidin gene expression is altered,

usually causing iron overload and leading to iron deposition in the liver, as well as higher levels of non-transferrin-bound iron in the bloodstream." (see Page 14, line 22-24)

Response to Reviewer B

The authors tested the effects of intravenous ferric carboxymaltose supplementation in a rat model of liver cirrhosis. The authors report that iron supplementation had little effect on markers of liver dysfunction or markers of iron status, albeit some inflammatory and oxidative stress markers were lower in the iron-treated group. The authors conclude that intravenous iron supplementation appears safe and beneficial in reducing inflammation and oxidative stress in this disease model.

Major Comments.

Comment 1: A major limitation of the study is that the animal model does not develop signs of overt anemia. Although the authors report that "anemia was observed in both the LC and LC-iron groups", anemia is typically defined by Hb levels below 110g/L, rather than 130g/L. Irrespective of the definition of anemia used, it would seem that the hematological effects of CCl4 treatment would not be severe enough to warrant intravenous iron therapy in a patient, and therefore the validity of the study is questionable.

Reply 1: Thank you for your comment. As you mentioned, the impact of iron therapy

is not remarkable in the present study. However, to test safety of iron administration was our primary outcome and relatively lacked anemic level. Accordingly, we would not expect iron administration to have a major impact on the anemia since this is not a model that causes iron deficiency. In terms of the definition of anemia, the World Health Organization (WHO) defined anemia as hemoglobin (Hb) concentration < 130g/L in male and < 120g/L in female. In the perspective of patient blood management, numerous previous literatures adapted this definition for diagnosis of anemia.

It is common that high frequency of hematological abnormalities is followed by advanced liver disease. Iron deficiency anemia is frequent among complications of chronic liver disease. Since the liver plays a major role in iron homeostasis, it is clear that liver disease with various etiology are directly related to alteration of iron homeostasis, which makes the diagnosis of iron deficiency anemia very difficult. The superiority of ferritin over other parameters to diagnose iron deficiency has been proven by several studies, so ferritin level is commonly used. However, the fact that ferritin is an acute phase protein, affected not only by the iron status of the body, but also by acute or chronic inflammation, malignancies and liver disease, makes the interpretation of test results challenging. According to the recent study, in the general population, a cut off value of ferritin to diagnose iron deficiency is < 12 μ/L. However, when it comes to patients with inflammation of chronic liver disease, the level is significantly higher with the best predictor being < 100 μ/L (or higher) [1, 2]. In systemic analysis, it was found that measurement of serum ferritin was particularly helpful, even in a population with chronic liver disease or inflammation, but the test results in these patients needs to be

interpreted with a different cut off value as mentioned above [3]. According to above described evidence, elevated ferritin level of our study in LC induced rats comparted to control group is explained by the inflammatory state by liver disease. Accordingly, the cut-off value of ferritin also should be interpreted based on disease state of rats, which shows that rats in control group are not in the iron deficiency (ferritin > 12 μ/L) and rats in LC groups are in the iron deficiency (ferritin < 100 μ/L). Thus, treatment of iron deficiency can be useful in rats with liver cirrhosis, even the ferritin level was higher than the limit range of healthy population.[4]

Moreover, recently, numerous evidence support the importance of treating functional iron deficiency.[5-7] In inflammatory states by liver disease, although serum levels of ferritin are high, iron is unable to circulate or be delivered to the bone marrow, which in turn leads to iron-restricted erythropoiesis.[2, 8] Therefore, in this state, elevated serum ferritin and iron deficiency may sell coexist. Previous study reported by Rossler et al. have made the evidence highlighting that preoperative iron deficiency, even in the absence of anemi,a is an important prognostic marker for postoperative mortality.[6]

Because to test safety of iron administration was our primary outcome, we would not expect iron administration to have a major impact on the anemia without iron deficiency rat model. However, recent evidence of importance of absolute and functional iron deficiency in both anemic and non-anemic patients may validate the findings of the study.

Changes in the text: “However, we would not expect iron administration to have a

major impact on the anemia since this is not a model that causes iron deficiency. In inflammatory states by liver disease, although serum levels of ferritin are high, iron is unable to circulate or be delivered to the bone marrow, which in turn leads to iron-restricted erythropoiesis (30,31). Previous study have made the evidence highlighting that preoperative iron deficiency, even in the absence of anemi,a is an important prognostic marker for postoperative mortality (32). Recent evidence of importance of absolute and functional iron deficiency in both anemic and non-anemic patients may validate the findings of the study." (see Page 17, line 13-20)

Comment 2: Another limitation is the use of only a single dose of intravenous iron carboxymaltose. It is premature to conclude that treatment with ferric carboxymaltose is safe in this model, when higher doses have not been tested. Although the authors state that the dose used is recommended by the manufacturer, it is not clear whether this recommendation is applicable to rodents. Moreover, since there was no overt anemia in the model, it's not clear if a dose of 20mg/kg would be effective in treating anemia or iron deficiency. In fact, in ref, 10, Toblli et al reported deleterious outcomes with carboxymalrose injection at doses of 40mg/kg.

Reply 2: We totally agree with your concern about single dose of intravenous ferric carboxymaltose (FCM). As you mentioned, there are possibilities that higher doses of IV iron can cause deleterious outcomes, so additional experiment using higher doses should be tested to conclude that treatment with FCM is safe. In line with this concern, we are planning to conduct additional experiments with higher dose of FCM and our

pilot studies showed that, even in groups with higher dose, the safety of IV iron was observed similar with the present study. This experiment will be our next topic for upcoming article, so we couldn't include the results of this pilot study. We also mentioned this issue in limitation section as below.

Changes in the text: "Second, we used only one Ferinject dose of 20 mg/kg, which is the standard dose recommended by the manufacturer. Thus, further experiments using various doses in a rat model of cirrhosis are needed to identify and confirm safe and effective dosing levels." (see Page 19, line 5-8)

Comment 3: It's difficult to get a sense of the degree of iron deficiency in this model, because inflammatory markers are known to influence hepcidin levels, and in turn affect biomarkers of iron homeostasis. Presumably, we would anticipate lower hepcidin levels in those with more pronounced depletion of iron, yet hepcidin levels are markedly elevated in LC and LC-iron groups. One of the few markers that is minimally affected by inflammation is the soluble transferrin receptor, but this was not assessed herein.

Reply 3: We appreciate your constructive comment. As you mentioned, we agree with the difficulty of interpretation of iron profile in patients with liver cirrhosis. Because of the inflammatory status induced by liver disease, Hepcidin level can be naturally affected. Previous study revealed that mean Hepcidin level was higher in patients with chronic liver disease such as hepatitis B or hepatocellular carcinoma, compared with the healthy population.[4], which can explain the marked elevation of Hepcidin level

in LC and LC-iron groups compared to control group. Accordingly, we, rather, focused on the difference between LC and LC-iron groups and discussed the significant difference of Hepcidin level between two groups. Unfortunately, we did not test the soluble transferrin receptor and this is one of our study's limitations. We added this content in the limitation section.

Changes in the text:

". In our analysis, the hepcidin level was significantly greater in the LC-iron group than in the LC group, which can be explained by natural reaction following high dose of iron injection." (see Page 16, line 14-15)

"Because inflammatory state following liver cirrhosis is known to alter the iron related biomarkers, markers that is minimally affected by inflammation, such as the soluble transferrin receptor, can be used for test." (see Page 19, line 2-4)

Comment 4: Abstract – Conclusion is simply a reiteration of the findings presented in prior section.

Reply 4: Thank you for your comment, according to your comment, we revised conclusion in the abstract.

Changes in the text: "Although the administration of intravenous iron appears safe in our rat model of cirrhosis, further preclinical and clinical studies are warranted to confirm the safety and efficacy of intravenous iron in patients with liver cirrhosis or end-stage liver disease." (see Page 3 ,line 21-23)

Comment 5: The description of the experiments and use of animals is confusing. It would be helpful if the authors described the methods and results as two distinct studies (i.e. a pilot and a full study), and describe the number of animals, assays employed, and statistical tests used in each.

Reply 5: We agree with your comment. To clarify the methodology, we added a diagram including the number of animals and assays employed. We marked the process of pilot and main experiments. Following your recommendation, we described the result as two distinct studies (i.e. a pilot and a main experiment).

Changes in the text: Please see the diagram (Figure 1).

"Pilot study to Confirm of liver fibrosis

Ten weeks after the initiation of CCl4 administration, AST levels were significantly higher in the LC (443 ± 110 IU/L) and LC-iron (444 ± 117 IU/L) groups than in the control group (69 ± 7 IU/L, P<0.001). ALT levels were also significantly higher in the LC (518 ± 186 IU/L) and LC-iron (520 ± 252 IU/L) groups than in the control group (91 ± 7 IU/L, P<0.001). Histological analyses showed disrupted liver architecture with fibrotic bands, parenchymal nodules, and vascular distortion, which implied liver cirrhosis. Sirius red staining showed significantly greater levels of fibrosis in the LC and LC-iron groups than in the control group.

Main experiment: Liver fibrosis and iron deposition

Histological examination indicated that after CCL4 administration, there were significantly greater degrees of fibrosis in the LC and LC-iron groups than in the control

group (Figure 2A). The level of iron accumulation was significantly greater in the LC-iron group than in the LC group at all measurement points (Figure 2B). The degrees of fibrosis were comparable between the LC-iron and LC groups on days 2, 14, and 28 (P=1.0, P=0.25, and P=1.0, respectively). The degree of hepatic iron accumulation was significantly greater in the LC-iron group than in the LC group on days 2 (P<0.001), 14 (P=0.029), and 28 (P<0.01). Histopathological examination indicated ballooning cells, fatty changes, fibrosis, and iron accumulation in liver tissue (Figure 2). Focal iron accumulation was observed in the portal triad in the LC group, but diffuse accumulation was present in the hepatic triad and liver lobules in the LC-iron group. Iron deposition (visualized using Prussian blue staining) was mainly deposited to reticuloendothelial cells in the LC-iron group but showed mixed distribution in non-reticuloendothelial hepatocytes and reticuloendothelial cells in LC group." (see Page 10, line 14 – Page 11, line 21)

Comment 6: (1) Several assays are mentioned but not described. Pg. 7, line 1: how was blood collected? Pg7, line 2-3: how were AST and ALT assessed?

(2) The statistical approach is not clear. The authors describe using both repeated measures ANOVA with Bonferroni post hoc test, as well as one-way ANOVA with Dunnett's multiple comparison test, though its not clear which data sets were analyzed by each method.

Reply 6: (1) Thank you for your comment, following your comment we revised manuscript as below. (2) We appreciate your important comment. To avoid confusion,

we changed the manuscript as follow.

Changes in the text: (1) "Blood samples were collected through the tail vein and serum was centrifuged at 3000 rpm for 10 minutes, and AST and ALT levels were measured using a blood biochemical analyzer and an automatic analyzer." (see Page 7, line 7-9)

(2) "For assessing differences among groups, data with a normal distribution were compared using One-way analysis of variance with the Bonferroni post hoc test and data without a normal distribution were compared using the Friedman test with the Dunn post hoc test." (see Page 10, line 11-14)

Comment 7: It's not clear why the control group data is not shown in Figure 1?

Reply 7: Thank you for your comment. Because the level of LFT was negligible and can not be showed in the graph to put together with LC and LC-iron group. Moreover including control group can distract readers so we did not show the control group in the figure 1. Instead of showing the control group, we described the reason why the control group data is not shown in Figure 2 (original Figure 1).

Changes in the text: "Mean AST and ALT levels of Control group throughout the main experiment were 98.4 ± 14.9 IU/L and 75.9 ± 12.7 IU/L, respectively. It was negligible to be showed in the graph to put together with LC and LC-iron group so we did not show the control group in the figure 4." (see Page 12, line 7-10)

Comment 8: The figure captions should contain sufficient detail to stand on their own. Groups and abbreviations should also be defined. What do the symbols denote? It

should clear that Day 2, 14, and 28 reflect times after infusion of iron, and not induction of cirrhosis.

Reply 8: Thank you for your constructive comment. Following your comment, we revised the figure captions t contain sufficient detail to stand on their own in each figure. As you mentioned, we added a diagram to clearly indicate that Day 2, 14, and 28 reflect times after infusion of iron, and not induction of cirrhosis.

Changes in the text: Please check the Figure legend and Figure 1~5.

"Figure Legend

Figure 1. Diagram of experimental protocol.

CCl4, carbon tetrachloride; LC, liver cirrhosis; Wk, week; Control group, normal, healthy rats without cirrhosis; LC group, liver cirrhosis induced rats continued to receive CCl4 twice weekly with intravenous saline; LC-iron group, liver cirrhosis induced rats continued to receive CCl4 twice weekly with administration of intravenous iron at day 0; Day 2, 14, and 28 reflect times after infusion of iron; Red arrow indicates CCl4 IP injection 2 times a week; Black arrow indicates the timing of blood sample for LFT; Purple arrow indicates the timing of blood sample for LFT and iron-related parameters; * indicates Autopsy for histopathologic exam

Figure 2. Histopathological findings (A) Liver fibrosis (hematoxylin–eosin staining, □100) and (B) Iron deposition (Prussian blue staining, □100).

(A) Liver cell fibrosis was scored from 0 to 4 (17): 0 = no sign of fibrosis or cirrhosis;

1 = enlarged, fibrotic portal tracts; 2 = periportal or portal–portal septa but intact architecture; 3 = fibrosis with architectural distortion but no obvious cirrhosis; and 4 = probable or definite cirrhosis.; (B) Histochemical localization in the liver tissue of each rat was calculated using a computer and image analyzer and expressed as the percentage of the area per millimeter of positive iron deposit staining.; Data are shown as means ± standard deviation.; Control group, normal, healthy rats without cirrhosis; LC group, liver cirrhosis induced rats continued to receive CCl4 twice weekly with intravenous saline; LC-iron group, liver cirrhosis induced rats continued to receive CCl4 twice weekly with administration of intravenous iron at day 0; Day 2, 14, and 28 reflect times after infusion of iron.; ***/** Significant difference at $p < 0.001$/$p < 0.01$ compared with the control group.

Figure 3. Measurement of portal vein pressure.

Data are shown as means ± standard deviation.; Control group, normal, healthy rats without cirrhosis; LC group, liver cirrhosis induced rats continued to receive CCl4 twice weekly with intravenous saline; LC-iron group, liver cirrhosis induced rats continued to receive CCl4 twice weekly with administration of intravenous iron at day 0; Day 2, 14, and 28 reflect times after infusion of iron.; ***/** Significant difference at $p < 0.001$/$p < 0.01$ compared with the control group.

Figure 4. Measurement of liver enzymes

Data are shown as means ± standard deviation.; AST, Aspartate transferase ; ALT,

Alanine transferase ; LC group, liver cirrhosis induced rats continued to receive CCl4 twice weekly with intravenous saline; LC-iron group, liver cirrhosis induced rats continued to receive CCl4 twice weekly with administration of intravenous iron at day 0; Day 2, 14, and 28 reflect times after infusion of iron.; ###/# Significant difference at $p < 0.001$/$p < 0.05$ compared with the LC group.

Figure 5. Measurement of levels of pro-inflammatory and oxidative stress markers. Data are shown as means ± standard deviation.; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; MDA, malondialdehyde; Control group, normal, healthy rats without cirrhosis; LC group, liver cirrhosis induced rats continued to receive CCl4 twice weekly with intravenous saline; LC-iron group, liver cirrhosis induced rats continued to receive CCl4 twice weekly with administration of intravenous iron at day 0; Day 2, 14, and 28 reflect times after infusion of iron.; ***/** Significant difference at $p < 0.001$/$p < 0.01$ compared with the control group.; ###/# Significant difference at $p < 0.001$/$p < 0.05$ compared with the LC-iron group." (see Page 27 – 28)