Peer Review File

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Reviewer A

The manuscript of Chun-Yan Ma et al titled "Berberine improves high-fat diet induced atherosclerosis and hepatic steastosis in ApoE$^{-/-}$ mice by down regulating PCSK9 via ERK1/2 pathway" focuses on the effects of Berberine on atherosclerosis and lipid accumulation in liver. Granted the study seems to uncover an interesting phenotype, some mechanistic aspects remain to be addressed to make the study solid.

Major comments:

Comment 1: Plaque analysis should also be performed in a second location, i.e., aortic roots, not just the aorta. Please also separate lesion size in thoracic aorta and aortic arch.
Reply 1: Thanks for the reviewer's scientific comments on this important issue. We cannot disagree with reviewer's kind suggestion that plaque analysis in a second location might be better; unfortunately, we had no such data available. Hence, we have discussed this issue in the section of discussion of the revised manuscript.
Changes in the text: There is no change in the revised manuscript.

Comment 2: The effects of BBR on atherosclerosis are extremely limited described. Please include results on the effects of BBR on lesion composition, like lesional macrophage, smooth muscle cell, collagen content and necrotic core. Are there less foam cells present in the lesions?
Reply 2: Thanks for the reviewer's academic and interesting comments. One of the limitations in the present study was not to determine the effects of BBR on lesion composition and the number of foam cells present in the lesions.
Changes in the text: There is no change in the revised manuscript.

Comment 3: In figure 3B it is not clear which graph represents Oil-Red-O and which one HE. The left graph states "arch", does this mean only the arch area was analyzed?
Reply 3: Yes, the figure 3B represented oil red O staining of the arch area. We are sorry for that the "arch" in the left graph was a mistake, and it should be "area" instead of "arch". We have revised these points in the new version. Both of the two graphs represented oil red O staining, which were relative lesion area (%) and lesion size (μm^2) respectively.
Changes in the text: Please see the Figure 3B in the revised manuscript.

Comment 4: Based on results shown in figure 4, the conclusion that Berberine

decreases hepatic steatosis is not justified as no quantification was performed and just representative pictures are shown. Please quantify the HE staining and oil red O staining to conclusively say that the BBR reduces lipid accumulation in liver. Additionally, measure liver cholesterol and triglyceride levels together with hepatic LDL levels to validate effects on liver lipids.

Reply 4: Thanks for the reviewer's kind suggestions. We have quantified the HE staining and oil red O staining (shown as Fig. 4B) and analyzed the effect of BBR on hepatic steatosis in ApoE$^{-/-}$ mice fed with HFD in the new version. In addition, our data showed that BBR treatment significantly decreased hepatic PCSK expression and increased LDLR level, which facilitating the metabolism of serum TC and TG.

Changes in the text: Please see the Figure 4B in the revised manuscript.

Comment 5: Does BBR affect systemic inflammation levels?

Reply 5: Thanks for the reviewer's scientific question. However, we did not preinstall an item regarding the effect of BBR on systemic inflammation due to the understandings of previous positive data (Int J Immunopathol Pharmacol 2019;33:2058738419866379; Acta Pharm Sin B 2020;10(9):1769-1783).

Changes in the text: There is no change in the revised manuscript.

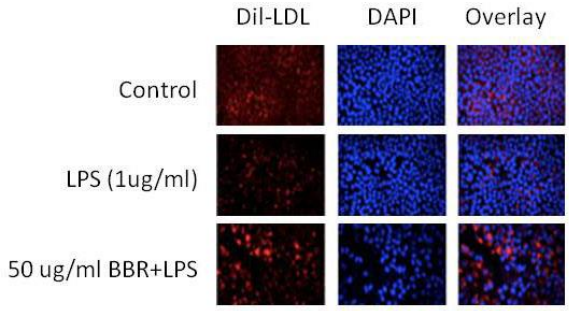
Comment 6: Is there an effect on systemic PCSK9 levels and PCSK9 levels inside the vessel/lesions? It is not clear whether the effects on the lesion are purely based on hepatic effects or whether also systemic/lesional effects occurred.

Reply 6: Thanks very much for the reviewer's interesting comments. However, in the present study, we didn't determine the systemic PCSK9 levels and PCSK9 levels inside the lesions.

Changes in the text: There is no change in the revised manuscript.

Comment 7: Is there an effect on lipid/fatty-acid uptake in the liver/HepG2 cells due to BBR treatment?

Reply 7: Thanks for the reviewer's scientific question. Our previous data showed that the treatment of BBR (50 ug/ml) could increase LDL uptake inhibited by LPS in HepG2 cells using fluorescence microscopy. The following figure was representative fluorescence microscopy images of cell-associated Dil-LDL (red), DAPI (blue) and the overlay.



Changes in the text: There is no change in the revised manuscript.

Comment 8: Berberine influences PCSK9 expression via ERK1/2 pathway is a novel finding. However, as the HepG2 cell line is not physiology, these key findings should be validated using primary hepatocytes. What about ERK1/2 expression in the liver of the mice?

Reply 8: Thanks for the reviewer's scientific comment. We agree completely with reviewer's opinion. However, HepG2 cell line has currently been widely used in lipid research fields for its characteristics of lipid metabolism and easily proliferation. We did not evaluate ERK1/2 expression in the liver of the mice. In vitro, we found BBR activated the ERK1/2 signal pathway by increasing the expression of phosphorylated ERK1/2.

Changes in the text: There is no change in the revised manuscript.

Minor comments:

Comment 1: Line 162 says low dose for 100 mg/kg/d Berberine, but it is supposed be written as high dose.

Reply 1: Thanks for your kindness. We have revised "low" to "high" in the new version.

Changes in the text: We have modified our text as advised (see Page 8, line 167) which is marked in red font.

Comment 2: The methods should state which exact statistical analysis were performed (outlier analysis, normality testing, which type of test)

Reply 2: We have revised the part of statistical analysis in the new version according to reviewers' kind suggestions.

Changes in the text: We have modified our text as advised (see Page11, line 253-259) which is marked in red font.

Comment 3: For the statistical analysis in figure 1, please explain clearly what groups you compare to bring about the p-values in the result section as well.

Reply 3: Thanks for your kind suggestion. We have described the differences and p-values between groups in the result section and in Figure Legend section respectively in the new version of the manuscript.

Changes in the text: We have modified our text as advised (see: Page 12, line 264-267;Page22, line 553-559) which is marked in red font.

Comment 4: We understand that the WT mice are 7 in number and ApoE$^{-/-}$ are 35 in number. Unfortunately, it is hard to understand what the n numbers are for each diet group. Please specify the n number in the methods section, results section as well as in the figure legends.
Reply 4: Thank you for your scientific suggestions. We are sorry for the carelessness of writing 28 as 35 in number. In the present study, the ApoE$^{-/-}$ mice are 28 in number, and there were 7 mice in number for each diet group. We have specified the n number in the methods section according to the reviewer's suggestion in the revised manuscript.
Changes in the text: We have modified our text as advised (see Page 7, line 140; page 7, line 162-167) which is marked in red font.

Reviewer B

General comment: Berberine is an interesting alkaloid that can be isolated from the medicinal plant. Several studies have shown that mice treated with Berberine have decreased levels of TC and TG and reduce atherosclerotic lesions. The mechanism for its effect is mostly unknown. The current study and others have tried to delineate the mechanism of berberine on its anti-atherosclerotic effect. The current study by Ma et al. demonstrated that the effect of berberine on reduction of atherosclerosis was through regulation of PCSK9 via ERK1/2 pathway. However, the mechanistic evidence was weak. It did not provide evidence on how berberine regulated PCSK9 levels. It did not provide reason on how ERK1/2 pathway was involved in the process. The English writing of this manuscript needs to improve, please ask an editor to help the writing.

Specific critics:

Comment 1: ApoE$^{-/-}$ mice should be written as Apoe$^{-/-}$ mice.
Reply 1: Thanks for reviewer's scientific suggestion. Although Apoe$^{-/-}$ mice may be exactly correct while the ApoE$^{-/-}$ mice is mostly used in the international peer-review journals (please kindly see: Atherosclerosis. 2015;238(2):321-328. Blood. 2016;127(21):2618-2629. Arterioscler Thromb Vasc Biol. 2017;37(2):237-246.). We would like to confer this issue to the editor which one is better.
Changes in the text: There is no change in the revised manuscript.

Comment 2: Why only male mice were used for the study? The authors need to provide an explanation.
Reply 2: There are two reasons for the male mice used in the present study. One is the

hormone and the other is due to previous references (please kindly see: J Nutr. 2020;150(2):249-255. Biomed Pharmacother. 2018;107:1556-1563.).
Changes in the text: There is no change in the revised manuscript.

Comment 3: On page 7, group 6 was listed in the method, but they were not studied? Please remove the group 6.
Reply 3: Thanks for reviewer's kind suggestion. We have removed the group 6 in the new version.
Changes in the text: We have modified our text as advised in the revised manuscript.

Comment 4: On page 7, line 166, the eyeballs were removed? This could not be an approved technique to collect blood? Please explain.
Reply 4: Thanks for reviewer's kind comment. We are sorry for our inaccurate description in the method part. Hence, we have revised the sentence of "The eyeballs were removed and blood samples were collected" into the sentence of "Blood samples were taken from the retro-orbital plexus and centrifuged" in the new version.
Changes in the text: We have modified our text as advised (see Page 8, line 169-170) which is marked in red font.

Comment 5: The quantification method on lesion measurement was missing in the method.
Reply 5: Thanks for reviewer's scientific suggestion. We have added the sentences about the quantification method on lesion measurement in the revised manuscript which are marked in red font.
Changes in the text: We have modified our text as advised (see Page 9, line 198-202) which is marked in red font.

Comment 6: The Fig3 of HE staining on aorta (line 255), which part of aorta? Usually, the investigator used aortic root section. The image on Fig3 does not look like aortic roots.
Reply 6: Thanks for reviewer's scientific question. We agree with reviewer's opinion that the investigator usually used aortic root section as histological analysis, while the part of aorta in Fig 3 by HE staining is aortic arch section.
Changes in the text: There is no change in the revised manuscript.

Comment 7: Why the Fig 4 of HE staining was so red?
Reply 7: Thanks for reviewer's scientific comment. The reason could be light dyeing of hematoxylin which resulted in the additional obvious red color of eosin during the procedures of Hematoxylin-eosin staining.
Changes in the text: There is no change in the revised manuscript.

Comment 8: There was no rational on why ERK1/2 pathway was studied?
Reply 8: Thanks for reviewer's scientific comment. It is well known that the MAPK/ERK1/2 signal pathway is involved in many cellular physiological processes and the expression of functional proteins. Our study detected that the phosphorylation of ERK1/2 was involved PCSK9 expression.
Changes in the text: There is no change in the revised manuscript.

Comment 9: Be careful on using "could" in your text in your result. Could indicate "possible", which the results show definite findings.
Reply 9: Thanks for reviewer's kind and scientific suggestions. We have revised these related sentences in the result part in our new version.
Changes in the text: We have modified our text as advised and revised the related words marked in red font throughout the new version.

Comment 10: How does berberine regulate PCSK9? PCSK9 synthesis?
Reply 10: Thanks for reviewer's scientific comment. Our data showed that berebrine reduced PCSK9 protein expression in HepG2 cells and decrease hepatic PCSK9 level.
Changes in the text: There is no change in the revised manuscript.

Comment 11: PCSK9 regulates LDLR levels not "density" (line 356).
Reply 11: Thanks for reviewer's scientific suggestion. We have revised the sentence of "PCSK9 is a key regulator of cholesterol homeostasis that controls LDLR density on the surface of hepatocytes" into the sentence of "PCSK9 is a key regulator of cholesterol homeostasis that controls LDLR levels on the surface of hepatocytes".
Changes in the text: We have modified our text as advised (see Page 16, line 374) which is marked in red font in the revised manuscript.

Comment 12: Did berberine use for clinic study? What was the finding?
Reply 12: Thanks for reviewer's scientific comments. Recently, a few clinical studies demonstrated that berberine improves lipid profiles in dyslipidemia with satisfactory safety. (Please kindly see: Efficacy and safety of berberine for dyslipidaemias: A systematic review and meta-analysis of randomized clinical trials. Phytomedicine. 2018;50:25-34).
Changes in the text: There is no change in the revised manuscript.

Comment 13: The study measured ABCA1, ABCG1 and SR-B1, but did not discuss the significance of these genes in association of this study.
Reply 13: Thanks for reviewer's scientific comments. The genes of ABCA1, ABCG1 and SR-B1 are involved in the process of reverse cholesterol transport which is

important for improving dyslipidemia. In the present study, data showed that berberine increased the protein expression of ABCA1, ABCG1 and SR-B1 in liver of ApoE-/- mice, which indicated berberine's beneficial effect on reverse cholesterol transport. We have added the significance of these results in discussion part in the revised version.
Changes in the text: We have modified our text as advised (see Page 17, line 389-392) which is marked in red font in the revised manuscript.

Reviewer C

Ma et al show that berberine reduces atherosclerosis and improves lipid profiles. This is associated with reduced PCSK9 in vivo. In vitro reductions in PCSK9 are associated with increased P-ERK. While interesting, these data do not add anything that is not currently known (in this model/dosing and cell type), and the mechanistic studies lack definitive causality.

Specific Comments:

Comment 1： Are these associated changes in ERK signaling seen the livers in vivo?
Reply 1： Thanks very much for reviewer's scientific and interesting comments. Unfortunately, we have not performed an analysis regarding ERK signaling in vivo.
Changes in the text: There is no change in the revised manuscript.

Comment 2： Does blocking this pathway (genetically or pharmacologically) prevent the effects of BBR to increase P-ERK and decrease PCSK9?
Reply 2： Thanks very much for reviewer's scientific suggestions. In vitro study, we used U0126, an inhibitor for ERK1/2 pathway, to investigate the effect of pharmacological blocking ERK1/2 pathway on PCK9 expression reduced by BBR. The data demonstrated that inhibiting ERK1/2 pathway resulted in an increase of PCSK9 expression in HepG2 cells, which indicated ERK1/2 pathway was involved in BBR-induced reduction of PCSK9 expression.
Changes in the text: There is no change in the revised manuscript.

Reviewer D

Comment 1： In my opinion, using anti-atherosclerosis is a very strong word. The author may consider using atherosclerosis protection or prevention.
Reply 1： Thanks for reviewer's kind and scientific comments on opinion of using anti-

atherosclerosis word, while this word for this drug study is extremely common (please kindly see papers: [1] Functional nano-vector boost anti-atherosclerosis efficacy of berberine in Apoe-/- mice. Acta Pharm Sin B. 2020;10(9):1769-1783. [2] Advance of studies on anti-atherosclerosis mechanism of berberine. Chin J Integr Med. 2010;16(2):188-192. [3] Comparative effect of berberine and its derivative 8-cetylberberine on attenuating atherosclerosis in ApoE -/- mice. Int Immunopharmacol. 2017;43:195-202).

Changes in the text: There is no change in the revised manuscript.

Comment 2： It is not common to use AS to abbreviate atherosclerosis. It is better to use atherosclerosis as a full term in the article.

Reply 2 We have revised "AS" into "atherosclerosis" throughout the new version according to reviewer's suggestions.

Changes in the text: We have modified our text as advised which is marked in red font in the revised manuscript.

Comment 3： Aim in the abstract is not specific, maybe only focus on the mechanism underlying the effect of BBR on atherosclerosis reduction.

Reply 3： Thanks for reviewer's scientific suggestion. We have revised the aim of the study in the new version.

Changes in the text: We have modified our text as advised (see: Page 3, line 53-55; Page 6, line 137-139;) which is marked in red font in the revised manuscript.

Comment 4： In the Abstract low dose of BBR was mentioned in both groups 4 and 5. Please correct them.

Reply 4： Thanks for reviewer's kind suggestion. We have revised "low" into "high" in group 5 in the new version.

Changes in the text: We have modified our text as advised (see Page 3, line 59-61) which is marked in red font in the revised manuscript.

Comment 5： In the result in the Abstract, please mention which dose of BBR reduced atherosclerosis.

Reply 5： Thanks for reviewer's scientific suggestion. We have added the dose of BBR which reduced atherosclerosis in the Abstract in the new version.

Changes in the text: We have modified our text as advised (see Page 4, line 71-74) which is marked in red font in the revised manuscript.

Comment 6： The author may consider using the English editor service to help to improve the manuscript.

Reply 6： We have re-written manuscript in English intensively and carefully according to your kind suggestion, which may be fulfilled with your journal's instructions.
Changes in the text: We have modified our text as advised in the revised manuscript.

Comment 7： In line 135, the aim of the study should be only to investigate potential mechanisms of BBR in atherosclerosis reduction. Because you mentioned that your previous finding has already shown the beneficial effect of BBR on atherosclerosis.
Reply 7： Thanks for reviewer's scientific suggestion. We have revised the aim of the study in the new version.
Changes in the text: We have modified our text as advised (see Page 6, line 137-139) which is marked in red font in the revised manuscript.

Comment 8： There is no group 6 in the Abstract and in the result. Please provide more information on why group 6 is not included in the Abstract and Result parts?
Reply 8： Thank you for reviewer's scientific suggestion. We are sorry for the carelessness of including group 6 in the Method part, which existed in the non-submission primary manuscript.
Changes in the text: We have modified our text as advised.

Comment 9： Please include a route of BBR administration in the Method, for example, ip, sc…
Reply 9： Thank you for reviewer's scientific suggestion. The route of BBR administration is i.g., and we have added it in the new version.
Changes in the text: We have modified our text as advised (see Page 8, line 167) which is marked in red font in the revised manuscript.

Comment 10： In line 166, please remove "The eyeballs were removed." but please specify which area that you collect the blood from, for example, Retro-orbital bleeding or venous sinus from the dorsal of the eyes.
Reply 10： Thank you for reviewer's scientific suggestion. We have revised the sentence of "The eyeballs were removed and blood samples were collected" into the sentence of "Blood samples were taken from the retro-orbital plexus and centrifuged".
Changes in the text: We have modified our text as advised (see Page 8, line 169-170) which is marked in red font in the revised manuscript.

Comment 11： Please provide more detail on how you do serum lipids analysis because the result is the most important result.
Reply 11： Thanks for reviewer's scientific suggestion. We have re-written the part of

serum lipids analysis in the new version. The details are as shown below: The LDL-C concentration was analyzed by a selective solubilization method (Low Density Lipid Cholesterol Test Kit, Kyowa Medex, Tokyo, Japan) with a coefficient of variation of<5% and a total imprecision of <10%. The high-density lipoprotein cholesterol (HDL-C) concentration was determined by a homogeneous method (Determiner L HDL, Kyowa Medex, Tokyo, Japan) with a coefficient of variation of <5% and a total imprecision of<10%. TC and TG free fatty acid levels were measured with enzymatic assay by the automatic biochemistry analyser (Hitachi 917, Tokyo, Japan).

Changes in the text: We have modified our text as advised (see Page 8, line 180-188) which is marked in red font in the revised manuscript.

Comment 12： Please provide information on the Pharmacokinetic of BBR. How did you pick the dose and half-life of it?

Reply 12： Thanks for reviewer's scientific comment. We have designed the study according to previous studies covering Pharmacokinetic of BBR and the reasons regarding dose selection and half-life information. (Please kindly see papers: [1] The metabolism of berberine and its contribution to the pharmacological effects. Drug Metab Rev. 2017;49(2):139-157. [2] Current knowledge and pharmacological profile of berberine: An update. Eur J Pharmacol. 2015;761:288-97. [3] Pharmacokinetic interactions between metformin and berberine in rats: Role of oral administration sequences and microbiota. Life Sci. 2019;235:116818.)

Changes in the text: There is no change in the revised manuscript.

Comment 13： In line 249, Instead of using "Effect of BBR based therapy on aorta pathology" you may consider using "BBR administration reduces atherosclerosis progression in ApoE-/- mice fed with high-fat diet".

Reply 13： Thanks for reviewer's kind suggestion. We have used "BBR administration reduced atherosclerosis progression in $ApoE^{-/-}$ mice fed with HFD" instead of "Effect of BBR based therapy on aorta pathology" in the new version.

Changes in the text: We have modified our text as advised (see Page 12, line 270-271) which is marked in red font in the revised manuscript.

Comment 14： In line 272, please change "Effect of BBR based therapy on lipid metabolism-related genes and proteins" to "BBR administration improves lipid metabolism-related genes and proteins in $ApoE^{-/-}$ mice".

Reply 14： Thanks for reviewer's scientific suggestion. We have changed "Effect of BBR based therapy on lipid metabolism-related genes and proteins" to "BBR improved the expression of lipid metabolism-related genes and proteins" in the new version.

Changes in the text: We have modified our text as advised (see Page 13, line 289-290)

which is marked in red font in the revised manuscript.

Comment 15： In line 396, the conclusion that more study is needed to further examine the potential mechanism….. In my opinion, it should be that the BBR improves hepatic lipid metabolism and resulting in a reduction of atherosclerosis in ApoE$^{-/-}$ mice.
Reply 15： Thanks very much for reviewer's scientific comment. We have revised this sentence in the revised manuscript.
Changes in the text: There is no change in the revised manuscript.

Comment 16： Figure 4, please provide a scale bar and more detail of the finding and how you identify the steatosis in the figure legend.
Reply 16： We have provided a scale bar in Figure 4 and quantified the hepatic steatosis in Figure 4B. We have added the details of the finding (in the Result part) and elucidated the characteristics of the steatosis in the figure legends in our new version.
Changes in the text: We have modified our text as advised (see: Page 12, line 283-288; Page 22, line 569-575; Page 24, line 613;) which is marked in red font in the revised manuscript.