Format for ANSWERING REVIEWERS

July 25, 2013

Dear Editor,

Thank you for giving us the opportunity to revise our manuscript. We appreciate thr encouragement and the constructive comments from the reviewers. We have revised our manuscript carefully, following the comments and suggestions. The revised sentences are marked in red in the revised manuscript. The detailed revisions are given in our Point-by-Point Response. We are now submitting the revised manuscript. We hope our revisions meet the approval of the reviewers.
Please contact us if you have any questions.
We look forward to hearing from you.
Thank you for your help.

Please find enclosed the edited manuscript in Word format (file name: 3146-review.doc).

Title: Electroacupuncture improves gut barrier dysfunction against prolonged hemorrhagic shock in rats

Author: Ming-Hua Du, Hong-Min Luo, Sen Hu, Yi Lv, Zhi-Long Lin, Li Ma

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 3416

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) reviewer 02497108:

The study is somewhat interesting.

We would like to thank the reviewer for the positive and constructive comments to this manuscript.

Additional information is required to strenghten the readability of the manuscript. Comments: 1. The study is performed to evaluate ST36 has any beneficial effects on hemorrhagic shock-induced intestinal barrier dysfunction. However, the information related to ST36 and intestinal barrier is not enough. Please provide additional information and cited references.

We have modified this and cited reference supporting that ST36 has beneficial effects on hemorrhagic shock-induced intestinal barrier dysfunction in the text.

Increasing evidences suggest that the effect of electroacupuncture at ST36 for gastrointestinal disorders may involve vagal reflex. The dorsal vagal complex (DVC) consists of the nucleus of the solitary tract (NTS), which receives primary visceral afferent information, and the dorsal motor nucleus of the vagus (DMNV), which contains the efferent vagal neurons innervating visceral organs. Therefore, the DVC plays an important role in regulating visceral functions. Substance P (SP) is a 13-amino acid polypeptide, which is widely present in DVC, and also plays an important role in modulating gastrointestinal functions. Previous study has demonstrated that there is a commonality of central nervous system (CNS) cell groups in brain controlling Zusanli (ST36) points, including dorsal motor nucleus of vagus nerve (DMV), nucleus tractus solitarius (NTS)(Lee CH, Jung HS, Lee TY, Lee SR, Yuk SW, Lee KG, Lee BH. Studies of the central neural pathways to the stomach

and Zusanli (ST36). Am J Chin Med. 2001;29(2):211-20.). Electroacupuncture at ST36 generated an increased expression of c-Fos expression in the neurons of DMV(Kim MH, Park YC, Namgung U. Acupuncture-stimulated activation of sensory neurons. J Acupunct Meridian Stud. 2012 Aug;5(4):148-55. doi: 10.1016/j.jams.2012.05.002.) and promoted the gastric myoelectric activity, which was regulated by the vagus, and SP in the DVC which involved in the excitatory effects (Liu JH, Yan J, Yi SX, Chang XR, Lin YP, Hu JM. Effects of electroacupuncture on gastric myoelectric activity and substance P in the dorsal vagal complex of rats. Neurosci Lett. 2004,356(2):99-102.). It has been proved that the changes of vagal electrical activity after EA at ST36, which proved that EA at ST36 can increase the eferent activity of the vagal nerve (Effect of Electroacupuncture at "Zusanli" (ST 36) on Vagal Electrical Activity in the Rat, Acupuncture Research, 2006; 31(5) : 290-293). Recently, researchers have demonstrated an expanded role for vagus nerve stimulation and the cholinergic anti-inflammatory mechanism that provides a protective effect on the gut against epithelial barrier dysfunction and alleviates inflammatory injury in intestine and remote organs. We have recently proved that EA ST36 can protect intestinal barrier integrity after intestinal ischemia and reperfusion injury, while surgical abdominal vagotomy eliminated the protective effects of ST36. Taken together, these data suggest that EA ST36 is relevant to vagus nerve and it has beneficial effects on intestinal barrier function.

2. ST36 in rat model- How to make sure the correct acupoint and proper depth?

We make sure acupuncture at ST36 acupoint with proper depth correctly and strictly firstly by reference to the latest standard chart of ccupuncture meridians and points in rats cited from experimental acupuncture science(Li Zhongren, experimental acupuncture science. Beijing: China Press of Traditional Chinese Medicine, 2007: 255-257), and secondly according to previous studies. Besides, our laboratory has been performing research concerning electroacupuncture at ST36 for almost 10 years; we can precisely perform acupuncture at the responding places in different rats.

3. The experimental design and timing may be not clear. The authors are suggested to provide a scheme for whole experiment.

We appreciate your suggestion, and we have modified this in the text.

4. Please provide information of ?-BGT.

We accept your comment, and we have modified this in the text. α-bungarotoxin(α -BGT) is an antagonist of α7 subunit of cholinergic nicotinic receptor, which inhibits the α7 subunit of acetylcholine receptors by blocking a pivotal communication pathway between the efferent vagus and intestinal immune cells. (Zhirong Gao, Mario H. Müller, Martina Karpitschka, Sarah Mittler, Michael S. Kasparek, Bernhard Renz, Andrej Sibaev, Jörg Glatzle, Yongyu Li, Martin E. Kreis. Role of the vagus nerve on the development of postoperative ileus. Langenbeck's Archives of Surgery 2010; 395:407-411.) (Marinou M, Tzartos SJ. Identification of regions involved in the binding of alpha-bungarotoxin to the human alpha7 neuronal nicotinic acetylcholine receptor using synthetic peptides. Biochem J. 2003;372(Pt 2):543-54.)

5. Page 3-“the vagus nerve can prevent cytokine release and tissue injury via an efferent neural signaling pathway termed the cholinergic anti-inflammatory pathway.” Please provide additional information related to electroaccupuncture and pathway mentioned above.

We accept your comment, and we provide additional information related to this in comment 1.

6. Methodology- Sham operation is lacking in vagotomy group. In addition, there is no vehicle in therapeutic drug group.

In this series of study, we had ‘control-normal’ group, ‘sham operation with vagotomy’ group and ‘control’ group corresponding to drug group, and the results showed that they had no positive influences. Duo to too many groups involved in this present manuscript, data of these groups are not shown in the manuscript.

7. Page 9- Please provide the possibilities of decreased TNF-a and IL-6 levels.

Previous studies have demonstrated that EA ST36 can antagonize systemic inflammatory response with no side effects. (Tian L, Huang YX, Tian M, Gao W, Chang Q. Downregulation of electroacupuncture at ST36 on TNF-alpha in rats with ulcerative colitis. World J Gastroenterol , 2003,9(5):1028-33.) (Scognamillo-Szabó MV, Bechara GH, Ferreira SH, Cunha FQ. Effect of various

acupuncture treatment protocols upon sepsis in Wistar rats. Ann N Y Acad Sci, 2004, 1026: 251-6.) (Yim YK, Lee H, Hong KE, Kim YI, Lee BR, Son CG, Kim JE. Electro-acupuncture at acupoint ST36 reduces inflammation and regulates immune activity in collagen-induced arthritic mice. Evidence-Based Complementary and AlternativeMedicine, 2007, 4(1):51–57.)

8. Please address the limitation of the study.

We appreciate your suggestion, and we have addressed the limitation of the study in the text.

9. Page 19- lines 8-10: "These results were in …IL-10 secretion" are suggested to deleted. In addition, please provide the meaning of "DAO activity".

We appreciate your suggestion, and we have modified this in the text.

10. Please correct some typos- For example: Page 3: We have demonstrated that EA at ST36 had a significant positive therapeutic effect on hemorrhagic shock [2] in rats with delayed fluid resuscitation of hemorrhgic shock, however, its mechanism remains unknown. Page 5: (a) EAN group: Rats were perfoemed EA at non-channel acupoints

We appreciate your suggestion, and we have modified this in the text.

(2) ***reviewer 02495050:***

HS causes multiple organ failure, and many studies tried to alleviate MOF in HS with various methods. However, nothing could be extended to clinical uses. In that sense, EA ST36 could be one simple methods, and it is very interesting its mechanism could be through vagus nerve.

We would like to thank the reviewer for the positive and constructive comments to this manuscript

Below are my comments. 1. First of all, English proofing is mandatory. Many grammatical errors are found, and this leads to poor understanding of your precious findings.

We appreciate your suggestion, and we have modified this in the text.

2. Introduction should be more organized.

We accept your comment, and we reorganized the introduction section.

3. In methods, it seems to be that volume controlled HS combined with pressure controlled HS was used. If right, please make it more clear.

In this study, hemorrhagic shock model was induced by volume controlled exsanguinations. Pressure controlled HS in the study was just to monitor the blood pressure of each animal, and we have modified it and made it clearer in the text.

4. In methods, is there sham group?

In this series of study, we had 'control-normal' group, 'sham operation with vagotomy' group and 'control' group corresponding to drug group, and the results showed that they had no positive influences. Duo to too many groups involved in this present manuscript, data of these groups are not shown in the manuscript.

5. In results, the last sentence in 3.3. section should go to discussion, or could be deleted if you think it is repetitive.

We accept your comment, and we have omitted the last sentence in 3.3. section.

6. EA group showed better hemodynamic results compared to other groups. Did you check NO or iNOS?

In this present study, we did not check NO or iNOS, but in our previous study, we have demonstrated that electroacupuncture at ST36 points had significant effects on promoting gastric emptying rate，inhibiting the contents of plasma NO after early oral fluid resuscitation of hemorrhagic shock in rats with 40% blood volume loss(Li-jian Zhang, Sen Hu, Jing-Yuan Hou, Guo-Yong Zhou, Xian Shi, Zhi-Yong Sheng. Effect of electro-acupuncture at Zusanli points on gastric em ptying and plasm a contents of NO and MTL during oral fluid resuscitation of hem orrhagic ShoCk in rats with bIood volume loss. World Chinese Journal of Digestology, 2009,17(4): 395-398).

7. How about showing the K-M curve with log rank test?

It may seem clearer with K-M curve, and in our previous studies concerning survival rate we always using K-M curve. In this study, regarding to the brevity of the experimental duration, we mainly focused on the first 12h after blood loss, and the survival rate were not so distinct among each group, so we only described it by word and figure.

8. In discussion, please spell out DAO.

We have modified this in the text.

9. In discussion, the following sentences seem to be irrelevant with this study; Moxibustion, one therapy of Traditional Chinese medicine has been reported to successfully repair tight junctions and enhance colonic epithelial barrier function in rats

We appreciate your suggestion, and we have omitted it.

(3) ***reviewer 00036765:***

The article by Ming-Hua Du et al. describes the protective effect of electroacupuncture in intestinal barrier dysfunction in an elegant animal model of hemorrhagic shock. The study in well designed and results seem to be very congruent among the different aspects of systemic inflammation and intestinal barrier function investigated.

We would like to thank the reviewer for your time, effort and constructive comments to this manuscript.

Major comments. 1. The authors conclude that the vagal nerve plays an important role in mediating this protective anti-inflammatory effect. Such effect has been described extensively in the literature. The question that remains unanswered by the authors is what the exact mechanism is that results in such activation of the vagal nerve through acupuncture. Release of certain hormones, such as CCK, has been suggested to play a role in the anti-inflammatory effect of vagal activation, but was apparently not investigated further in this study. The authors should provide more mechanistic insight into what happens between electrical stimulation of the ST36 point and the reinforcement of barrier function through vagal stimulation. The vagus has no receptive field in the tibial region, therefore, it is difficult for me to imagine such protective response without the release of either systemic mediators (CCK, for instance), or the involvement of neural circuits (involving the nucleus of the vagus nerve or the nucleus of the solitary tract, for instance). I suggest that the authors comment on this in the discussion.

We appreciate your suggestion, and we have modified this in the discussion. The relationship between vagus nerve and electroacupuncture at ST36 has been investigated and evaluated by several researchers. Increasing evidences suggest that the effect of electroacupuncture at ST36 for gastrointestinal disorders may involve vagal reflex. Previous study has demonstrated that there is a commonality of central nervous system (CNS) cell groups in brain controlling Zusanli (ST36) points, including dorsal motor nucleus of vagus nerve (DMV), nucleus tractus solitarius (NTS)(Lee CH, Jung HS, Lee TY, Lee SR, Yuk SW, Lee KG, Lee BH. Studies of the central neural pathways to the stomach and Zusanli (ST36). Am J Chin Med. 2001;29(2):211-20.). Electroacupuncture at ST36 generated an increased expression of c-Fos expression in the neurons of DMV(Kim MH, Park YC, Namgung U. Acupuncture-stimulated activation of sensory neurons. J Acupunct Meridian Stud. 2012 Aug;5(4):148-55. doi: 10.1016/j.jams.2012.05.002.) and promoted the gastric myoelectric activity, which was regulated by the vagus, and SP in the DVC which involved in the excitatory effects (Liu JH, Yan J, Yi SX, Chang XR, Lin YP, Hu JM. Effects of electroacupuncture on gastric myoelectric activity and substance P in the dorsal vagal complex of rats. Neurosci Lett. 2004,356(2):99-102.). It has been proved that the changes of vagal electrical activity after EA at ST36, which proved that EA at ST36 can increase the eferent activity of the vagal nerve (Effect of Electroacupuncture at "Zusanli" (ST 36) on Vagal Electrical Activity in the Rat, Acupuncture Research, 2006; 31(5) : 290-293). We have recently proved that EA ST36 can protect intestinal barrier integrity after intestinal ischemia and reperfusion injury, while surgical abdominal vagotomy eliminated the protective effects of ST36. But up to now, there is still no satisfactory conclusion and consensus about what exactly do acupuncture connects with vagus nerve.

2. The authors suggest that acupuncture could be an important tool in the emergency setting due to lack of resources of early fluid resuscitation. I assume however, although I am not familiar with the EA procedures itself, that EA also requires certain equipment, such as an EA apparatus, which may also

not be available in an emergency setting. I can hardly imagine that such apparatus is present, in for instance war setting, as the authors suggest. In case of a hypovolaemic shock, cardiopulmonary resuscitation is necessary to maintain circulation until the patient is transferred to a setting in which fluid resuscitation is available. Personally, I do not see a role for EA currently in the emergency situation. I would therefore suggest that the authors amend the discussion, as such statements divert the attention from the main message of the study.

In recent years, we have been working on the alternative methods to solve the complications caused by delayed fluid resuscitation until intravenous infusion fluids are available. We have proved that oral resuscitation is an effective way to partly replace immediate resuscitation when it is difficult to perform the intravenous infusion. And recently, we have proved that EA ST36 can significantly improve the survival rate and blood pressure after fatal hemorrhagic shock in rats. Both oral resuscitation and acupuncture are assistant measures to be performed until until intravenous infusion fluids are available. Besides, there are non-intravenous fluid infusion, life-sustaining drugs and cardiopulmonary resuscitation, which can also be used to maintain circulation until the patient is transferred to a setting in which fluid resuscitation is available.

We used electroacupuncture to perform more consistently reproducible interventions with same electrical current parameters among different groups in this animal experiment. While among treatment of patients in austere environments such as battlefield, earthquake, or accidents, manual manipulations can be used to avoid certain equipments, which would be more available in an emergency setting.

Minor comments 1. Please give a brief description of the ST36 points and the EA procedure. Not all readers are familiar with these.

We appreciate your comment, and we have described it and made it clearer in the text.

2. Please very briefly describe the vagotomy performed. What was the time interval between vagotomy, EA and hemorrhagic shock?

We appreciate your comment, and we have described it and made it clearer in the text.

3. Similary, please indicate the time interval between BGT treatment, EA and hemorrhagic shock. A schematic scheme of the treatments may be helpful.

We appreciate your comment, and we have modified it in the text.

4. I would suggest shortening the abstract to approximately 300 words or as otherwise instructed by the editorial guidelines.

We appreciate your suggestion, and we have modified it by the editorial guidelines.

5. Please spell out DAO in the discussion section.

We appreciate your comment, and we have modified it in the text.

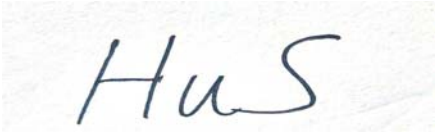
6. English language editing is necessary.

We appreciate your comment, and we have modified it in the text.

3 References and typesetting were corrected.

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,



Research Laboratory of Shock and Multiple Organ Dysfunction
Burns Institute
First Hospital Affiliated to the PLA General Hospital

No.51 Fu Cheng Road, Beijing 100048, China.
Telephone: +86-10-66867397 Fax: +86-10-68989139
E-mail: hs82080@yahoo.com.cn