Re: Manuscript number: 34554

Stable gastric pentadecapeptide BPC 157 in treatment of colitis and ischemia in rats. New insights

now revised as

Stable gastric pentadecapeptide BPC 157 in treatment of colitis and ischemia and reperfusion in rats. New insights

Dear Editor,

Thank you very much for your prompt response, and favorable comments given by the reviewers. It seems to us that all of the comments were adequate. Likewise, we strongly hope that the revised manuscript since completely rewritten, and much more focused, would satisfy and fully reply to all of the given arguments.

The following comments were raised by the Reviewer 1

> *In this manuscript the authors have investigated the effect of the gastric pentadecapeptide BCP 157 in a rat model of ischemic colitis. It is shown that BCP 157 protects from ischemia/reperfusion-induced endothelium damage. The cyto-protective effect of BCP 157 against colonic ischemia/reperfusion- mediated mucosal damage is associated with reduction of the oxidative stress and the normalization of NO synthesis. Data are convincing. However, the text needs lot of polishing. Sentences are too long and do not ease reading of the manuscript. The abstract can be shortened. Lengthy description of Materials and Methods is not needed. The results can be summarized with a few sentences highlighting key results. The discussion is too long and figure legends can be shortened.*

To the reviewer's comments see our arguments

> *In this manuscript the authors have investigated the effect of the gastric pentadecapeptide BCP 157 in a rat model of ischemic colitis. It is shown that BCP 157 protects from ischemia/reperfusion-induced endothelium damage. The cyto-protective effect of BCP 157 against colonic ischemia/reperfusion- mediated*

mucosal damage is associated with reduction of the oxidative stress and the normalization of NO synthesis. Data are convincing.

Acknowledged. We appreciate this point of the reviewer.

Sentences are too long and do not ease reading of the manuscript.

Acknowledged. As mentioned, the manuscript is now rewritten, and more focused, and sentences accommodated, not too long, and hopefully more suited for easy reading of the manuscript.

The abstract can be shortened.

Acknowledged. Abstract is to some extent shortened. However, considering the request given by another reviewer, new data were also incorporated. In addition, the size of the abstract is, at least partly, related to the requirements of the WJG.

On the other hand, Introduction was revised, and more focused, and shortened.

Lengthy description of Materials and Methods is not needed.

Acknowledged. Materials and Methods are suitably shortened. Details much more readily than before, are presented in the Figure 1.

The results can be summarized with a few sentences highlighting key results.

Acknowledged. In the concluding paragraph, we used most of the summary given by the reviewer.

In summary, these results show that BPC 157 protects colon tissue from the endothelial damage induced by ischemia and reperfusion. The cytoprotective effect of BPC 157 against colonic ischemia/reperfusion-mediated mucosal damage is associated with the activation of the collateral circulation, which circumvents obstructed sites and results in the resolution of the obstruction, reduction of oxidative stress and normalization of NO synthesis.

The discussion is too long and figure legends can be shortened.

Acknowledged. Discussion is completely rewritten, more focused, novel references included, and as requested, however, in accordance with the requests of other reviewers, incorporated with new items. This provides in particular inclusion of the new experiments, with removal of the ligations, thereby, inducing full reperfusion after the initial period of the deprivation of blood supply by two ligations, to clarify the issue of reperfusion. Likewise, figure legends are completely rewritten, clarified, and as requested, apparently shortened. However, as mentioned, the new figures were added to illustrate the requested clarification of reperfusion carried out in rats subjected to 15 minutes period of ligation and then 15 minutes of period of reperfusion.

In summary, we hope that the reviewer will be satisfied, and find that all comments are accordingly solved, and manuscript suited for final presentation in WJG.

Reviewer 2

The author describes protective effects of BPC 157 on ischemic injury of rat colon. The experimental model is sophisticated and findings are interesting.

Major comments

1. *The manuscript is too redundant. Especially, INTRODUCTION should be condensed to within 2 ~ 3 paragraphs (A4 double space, 3 pages). Conclusion of the end of DISCUSSION is also too long. Conclusion of DISCUSSION should be condensed to within 5 lines.*
2. *The author should consider ischemia and reperfusion separately. In the model of ischemic colitis, ischemic injury plays more important role rather than reperfusion injury. Reperfusion in this model is "recovery" but not "injury".*
3. *How does affect BPC 157 in healing of colon mucosa? What is the receptor of BPC 157 in colon? Author did not show precise mechanism of BPC 157. The author*

repeated importance of NO system in DISCUSSION. Protective effect of NO-system against ischemia/reperfusion injury is already shown in many organs more than 20 years ago. There is nothing new. The precise mechanism including VEGFR2 and VEGFR2-Akt-eNOS should be illustrated in figure.

4. *The manuscript must be revised by native English speaker.*
   Minor Comments

   1. Grammar errors; Especially, there are many space errors such as double or no space between words.

To the comments of the Reviewer 2 see our arguments

The author describes protective effects of BPC 157 on ischemic injury of rat colon. The experimental model is sophisticated and findings are interesting.

We appreciate this comment.

1. *The manuscript is too redundant. Especially, INTRODUCTION should be condensed to within 2 ~ 3 paragraphs (A4 double space, 3 pages). Conclusion of the end of DISCUSSION is also too long. Conclusion of DISCUSSION should be condensed to within 5 lines.*

Acknowledged. While the additional data were also incorporated, Introduction is condensed as requested. Likewise, conclusion of Discussion is also condensed.

2. *The author should consider ischemia and reperfusion separately. In the model of ischemic colitis, ischemic injury plays more important role rather than reperfusion injury. Reperfusion in this model is "recovery" but not "injury".*

Acknowledged. To clarify the issue that raised Reviewer 2, we performed additional experiments. In rats that had two ligations for a 15 minutes period, and then, after ligations were removed, and they were reperfused for next 15 minutes, BPC 157 bath was given at 1 minute of the reperfusion time. In addition to recording (USB microscope camera), the assessment of the MDA- and NO-colon tissue level was carried out at the end.

Thereby, ischemia and reperfusion were considered separately, and it was shown that BPC 157 beneficially affects either of them, providing that it was applied at 1

minute of the ligation time, or at 1 minute of reperfusion time. It seems to us that reperfusion while deprivation of blood supply is certainly present – should be taken as an innate one. The distinction with the repefusion that appears with removal of the obstruction was clearly emphasized in both Introduction, and Discussion. See in particular paragraph 1, and paragraph 2, Discussion

We demonstrated that well-placed arcade vessels respond poorly to the increased demands that occur upon blood supply deprivation, reperfusion or additional bowel obstruction. Thus, the particular susceptibility of the colon to insufficient vascular perfusion [1] mandates that the main focus of the treatment of such conditions should be bypassing one or more of the vascular obstructions. Then, the main focus is maintaining vessel function upon the first innate reperfusion (as evidenced by an initial innate recovery while the blood supply is deprived) in IC rats (which is also applicable much later with additional bowel obstruction (IC+OB rats)), and subsequently upon massive reperfusion following the removal of vascular obstruction(s) (IC+RL rats).

Based on this reasoning, therapy for ischemic colitis was administered once at early ligation-time (IC-rats) or alternatively at early post-ligation-time (IC+RL rats). In some animals, the therapy was administered once at a later time after additional colon obstruction (IC+OB rats). BPC 157 therapy was shown to cure rat ischemic colitis in both the very early and late time points and under diverse harmful conditions (short-lasting blood deprivation (IC rats) vs. reperfusion (IC+RL rats) vs. long-lasting blood deprivation and additional bowel obstruction (IC+OB rats)).

In addition, several additional Figures were included to illustrate particular effect going on during reperfusion after removal of the obstructions.

3. *How does affect BPC 157 in healing of colon mucosa? What is the receptor of BPC 157 in colon? Author did not show precise mechanism of BPC 157. The author repeated importance of NO system in DISCUSSION. Protective effect of NO-system against ischemia/reperfusion injury is already shown in many organs more than 20 years ago. There is nothing new. The precise mechanism*

including VEGFR2 and VEGFR2-Akt-eNOS should be illustrated in figure.

Acknowledged. We should however object the Reviewer 2 statement „*Protective effect of NO-system against ischemia/reperfusion injury is already shown in many organs more than 20 years ago.*“ To this point see our initial statment already emphasized in previous Introduction

> **Of note, while NO-system is largely implicated in stomach cytoprotection as well as in colitis lesion [1-3], however, the application of L-NAME (vasoconstrictory) [38] and/or L-arginine (vasodilatory) [39] was not investigated on the immediate presentation of the blood vessels going on after a segment of left colic artery and vein was excluded by two ligations. On the other hand, BPC 157 largely interacts with NO-system, in various models and species, in cytoprotection studies, in particular, carried out in studies using both L-NAME and L-arginine, as the individual agents or combined [1-3].**

This point is now reemphasized obstruction was clearly emphasized in both Introduction, and Discussion

> *Notably, although the NO system is largely implicated in stomach cytoprotection and colitis lesions [2-4], the application of L-NAME (a vasoconstrictor) [39] and/or L-arginine (a vasodilator) [39] has not been investigated with respect to the immediate presentation of the blood vessels after a segment of left colic artery and vein was occluded by two ligations. By contrast, BPC 157 largely interacts with the NO system in various models and species, as shown in cytoprotection studies, in particular, studies using both L-NAME and L-arginine as individual agents or in combination [2-4].*

Thus, this is a new point. Also, an important and new finding is that neither of them, L-NAME and L-argine, was effective, rather, both induced lesions aggravation. Given as the individual agents, both are acting as a NO-agents, since when given together, they antagonized each other effect. Thereby, in these terms, the interaction

of BPC 157 with NO-system, is certainly worthy, and certainly, it might provide some insight into the mechanism of BPC 157 beneficial effect (that was termed „consolidation of NO-system stimulating and inhibiting effects towards more healing effectiveness (i.e. interconnected arcade vessels to bypass major obstructions)" – based on the evidence that BPC 157 counteracted effect of L-NAME, as well as that BPC 157 counteracted effect of L-arginine. The supportive evidence was also given that BPC 157 might accordingly counteract the other effects of L-NAME as well as the other effects of L-arginine in different models as well.
And finally, there are only rare studies carried out with simultaneous application of L-NAME and L-arginine as individual agents, as well as in combination. Commonly, only L-NAME administration was used to demonstrate the involvement of NO-system.
Therefore, this is not only a repetition, since this study reveals an importance of NO-system for the essential early events, so far not described in the literature.
These were already mentioned in the previous version, as well as again reemphasized in Discussion (see paragraphs 6-10)
We hope that the Reviewer 2 will appreciate our arguments.

The question of the receptor of BPC 157 in colon was mentioned in the previous version (see Discussion, paragraph 7 and paragraph 9). Now, this point is much more elaborated (see Discussion, paragraph 10, paragraph 11).

We also fully appreciate the suggestion that precise mechanism should be illustrated in the figure. However, the main focus of this study was to demonstrate an essential phenomenon, so far not investigated, that would be however essential for the healing process. This point is now reemphasized in Introduction and Discussion already in the introductory paragraphs (see Discussion paragraph 1)

> *We demonstrated that well-placed arcade vessels respond poorly to the increased demands that occur upon blood supply deprivation, reperfusion or additional bowel obstruction. Thus, the particular susceptibility of the colon to insufficient vascular*

perfusion [1] mandates that the main focus of the treatment of such conditions should be bypassing one or more of the vascular obstructions. Then, the main focus is maintaining vessel function upon the first innate reperfusion (as evidenced by an initial innate recovery while the blood supply is deprived) in IC rats (which is also applicable much later with additional bowel obstruction (IC+OB rats)), and subsequently upon massive reperfusion following the removal of vascular obstruction(s) (IC+RL rats).

The next focus was demonstration that an agent with essential cytoprotective capability of protection of endothelium may do so, making essential cytoprotection endothelium protection suited for more complex circumstances, ischemia and reperfusion in ischemic colitis model. Thereby, the precise mechanism (beyond that explored in the present study by application of NO-agents, L-NAME and/or L-arginine, as individual agents, and in combination, MDA- and NO-tissue levels determination in both blood deprivation and full reperfusion conditions) should remain for the further studies. Finally, the complexity of the problem at the molecular pathways level was specially elaborated in the paragraph 11 of Discussion. We hope that Reviewer 2 will accept our arguments.

4. The manuscript must be revised by native English speaker.

Minor Comments

1. Grammar errors; Especially, there are many space errors such as double or no space between words.

Acknowleded. The manuscript was revised by native English speaker, and grammar errors and space errors were corrected.

Reviewer 3

A tight and simple presentation of the experimental procedure and discussion would help to facilitate understanding of obtaining results and especially to understand what's new in cytoprotection of pentadecapeptide BPC 157.

1. *The presented document from the Local Ethical Committee for experiments with animals is out of date (2007 – 2011)! And showed a decision covering the handling of animals from wide range of experimenters (1400 rats?). At least this is not in accordance with the new lines of experimental pharmacology to reduce the number of experimental animals.*
2. *"Aim: Stomach cytoprotection/ischemic colitis lesion may have analogous therapy, thereby, prototype cytoprotective agent (CA), gastric pentadecapeptide BPC 157." Please, change the expression in a way to be more convincing the argument and purpose of the study.*
3. ***Methods** - MDA determination: Some important details from the experimental procedure are missing when described the MDA determination in tissue samples (tissue homogenization medium, time of boiling – 60 min?, TCA concentration, post-boiling centrifugation).*
4. ***Discussion**: MDA as indicator of oxidative stress after 15 min tissue oxygen deprivation is not relevant parameter. Because oxidative stress started after the re-oxygenation and the first indices of oxidative stress could not be seen so quickly. The TBARS reactive products (MDA) are late breakdown products of heavy cytotoxicity and lipid peroxidation.*
5. ***Conclusion**: In this context it is sufficient the authors stressed that the cytoprotection of colon mucosa by BCP is due to the possibilities of enhanced collateral vascularization, instead of antioxidant effects.*

To the comments given by the Reviewer 3 see our arguments

> *A tight and simple presentation of the experimental procedure and discussion would help to facilitate understanding of obtaining results and especially to understand what's new in cytoprotection of pentadecapeptide BPC 157.*

We appreciate this comment. To do this, the whole manuscript is completely rewritten, and hopefully, these points accordingly emphasized and clarified.

1. *The presented document from the Local Ethical Committee for experiments with animals is out of date (2007 – 2011)! And showed a decision covering the handling of animals from wide range of experimenters (1400 rats?). At least this is not in accordance with the new lines of experimental pharmacology to reduce the number of experimental animals.*

We appreciate this concern. However, in general, our experiments are performed in accordance with new lines of experimental pharmacology to reduce the number of experimental animals (for details see our recent paper PLoS One. 2016 Sep 14;11(9):e0162590).

2. *"Aim: Stomach cytoprotection/ischemic colitis lesion may have analogous therapy, thereby, prototype cytoprotective agent (CA), gastric pentadecapeptide BPC 157." Please, change the expression in a way to be more convincing the argument and purpose of the study.*

Acknowledged. To this comment of the Reviewer 3, see revised Introduction, paragraphs 1-5.

3. ***Methods*** *- MDA determination: Some important details from the experimental procedure are missing when described the MDA determination in tissue samples (tissue homogenization medium, time of boiling – 60 min?, TCA concentration, post-boiling centrifugation).*

Part of the section on the determination of MDA has been revised according to reviewers comments. The information on the homogenization medium (PBS supplemented with 0.1 mM BHT) was added along with other relevant information regarding parameters used throughout experiments performed.

This subheading now reads:

> *At the end of the experiment and at 15 minutes of ligation time or at 15 minutes reperfusion time, oxidative stress in the collected tissue samples was assessed by quantifying thiobarbituric acid-reactive species (TBARS) as malonedialdehyde (MDA) equivalents. The tissue samples were homogenized in PBS (pH 7.4) containing 0.1 mM butylated hydroxytoluene (BHT) (TissueRuptor, Qiagen, USA) and sonicated for 30 sec in an ice bath (Ultrasonic bath, Branson, USA). Trichloroacetic acid (TCA, 10%) was added*

to the homogenate, the mixture was centrifuged at 3,000 rpm for 5 min, and the supernatant was collected. Then, 1% TBA was added, and the samples were boiled (95°C, 60 min). The tubes were then kept on ice for 10 minutes. Following centrifugation (14,000 rpm, 10 min), the absorbance of the mixture at the wavelength of 532 nm was determined. The concentration of MDA was read from a standard calibration curve plotted using 1,1,3,3′-tetraethoxy propane (TEP). The extent of lipid peroxidation was expressed as MDA using a molar extinction coefficient for MDA of 1.56 × 105 mol/L/cm. The protein concentration was determined using a commercial kit. The results are expressed in nmol per mg of protein.

4. ***Discussion****: MDA as indicator of oxidative stress after 15 min tissue oxygen deprivation is not relevant parameter. Because oxidative stress started after the re-oxygenation and the first indices of oxidative stress could not be seen so quickly. The TBARS reactive products (MDA) are late breakdown products of heavy cytotoxicity and lipid peroxidation.*

Acknowledged. Although in an other context, this point was mentioned by the Reviewer 2, and we provided a large explication. Briefly, we performed additional experiments. In rats that had two ligations for a 15 minutes period, and then, after ligations were removed, and they were reperfused for next 15 minutes, BPC 157 bath was given at 1 minute of the reperfusion time. In addition to recording (USB microscope camera), the assessment of the MDA- and NO-colon tissue level was carried out at the end. We hope that these additional data (correlating vessels presentation, mucosal lesions, gross and microscopy assessment, and MDA-tissue level increase) will provide a additional convincing background for the comments that we made in previous version of manuscript, and resolve this comment of the Reviewer 3. To additional support, we cited our most recent paper Inflammopharmacology. 2017;25:255-264.

5. ***Conclusion****: In this context it is sufficient the authors stressed that the cytoprotection of colon mucosa by BCP is due to the possibilities of enhanced collateral vascularization, instead of antioxidant effects.*

We full acknowledged this point. See our concluding paragraph in Discussion.

Finally, the aforementioned rapid and successful recruitment of the blood vessels during harmful events suggests that the application of BPC 157 may offer a fundamental treatment by providing the cytoprotection/endothelium protection that is essential [2-8,11-16] to quickly restore blood supply to the ischemically injured area and rapidly activate collaterals during various harmful conditions such as vascular obstruction, short-lasting blood deprivation, reperfusion, long-lasting blood deprivation and additional bowel obstruction.

We hope that we fully accepted and incorporated all of the comments given by the reviewers. Finally, considering the comments of the Reviewer 2, in particular, we carried additional studies to demonstrate separate effect on ischemia and reperfusion. Due to these improvements, and text modification, we would suggest modification of the title (*Stable gastric pentadecapeptide BPC 157 in treatment of colitis and ischemia in rats. New insights*). The new title should be *Stable gastric pentadecapeptide BPC 157 in treatment of colitis and ischemia and reperfusion in rats. New insights*

Sincerely

Predrag Sikiric, MD, PhD

Professor