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RE: Submission: "In Vivo Analysis of Intestinal Permeability following Hemorrhagic Shock"
Number ID: 03346858

Dear Editors,

Thank you for your review of the above manuscript. The reviewers' comments (underlined) have been reviewed and our responses to each point are as below and changed in the manuscript, as applicable. Changes in the content of the manuscript are in *track changes*. Because of the substantial formatting changes to the manuscript (but not to content), formatting changes are not in track changes in order to increase readability.

Reviewer 1:
…I wonder why the differences between the two groups regarding systemic concentrations of proteolytically-generated peptides from fluorescently labelled casein (fig. 1) were only statistically different in the first 40 minutes. What do you think it happened between the minute 40 and the reperfusion time? Do you think that is some kind of mucosa adaptation or something related to the experimental protocol? As you say, low flow due to hypotension can underestimate theses values; even so, it is interesting to see that the fluorescence drops from minute 40 to 100 - maybe because a more severe lower blood flow state as the organism is subject to a more time of hypotension? So, can we think that the more detrimental translocation occurs in the first minutes after a major bleeding, and after 20 minutes it losses relevance? If this is the case, we can think that translocation is not important for the perpetuation of the shock, since it is only significant in the first minutes. What is your opinion?

This is an excellent question and astute observation. The increase in fluorescence at reperfusion is easier to answer and is addressed in the Discussion: this is probably due to either reperfusion injury and/or increased circulation in the microvasculature commensurate with increased perfusion and return of blood pressure. Why there is a decrease in the systemic concentrations of labeled peptides at 40 minutes is unclear to us. A possible explanation is decreased microcirculatory and thus bulk flow from the bowel that lags systemic pressure decreases (anecdotal observations of the mesenteric microcirculation). Other possible explanations include mucosal adaptation [the time-constant for bulk release of enterocyte mucin in response to adverse stimulus is on the order of several minutes] (a decrease in delivery) and/or an initial increase in (hepatic?) clearance. Because these explanations are more

conjecture than anything, they were not included in the text, but are certainly fascinating and worthy of further study.

The question about the relevance of translocation after initial major bleeding is also fascinating. To answer this question it would be necessary to repeat this study without a reperfusion period. Our best guess is that at some point frank necrosis of the bowel wall will allow a further increase in translocated peptides. We do not think that at this time a definitive statement can be made about timing and relative importance of permeability changes; this is certainly a provocative and interesting point.

Also, in the manuscript you always talk about hemorrhagic shock in general. Do you think that the results will be reproducible if the cause of the hemorrhagic shock was an upper GI bleeding, with the consequent large amount of blood in the small bowel? Do you think that the results would be even more different between the two groups due to the large amount of protein in the lumen?

The underlying mechanisms operant in the egress of proteolytically-derived peptides to the systemic circulation are two steps: firstly, an ischemic event to the (small) bowel, whose capillaries are quite susceptible to ischemia. Therefore, any low-flow condition can precipitate this: hemorrhagic shock, septic shock, GI bleed resulting in hypoperfusion to the bowel, etc. The postulated immediate mechanism is a decrease in ATP necessary to produce proteolytically impermeable mucin for the mucus layer overlying the bowel. Once this is compromised, a second process, direct proteolytic action on the underlying mucosa itself, leads to rapid increases in permeability.

GI bleeding without small bowel malperfusion, and assuming the pressure gradient is forcing blood *into* the bowel, would probably not lead to marked permeability increases despite the protein load because: a) the pressure gradient is forcing blood into the bowel, not from the bowel, b) blood is relatively well-oxygenated, at least compared to bowel contents (assumption is $0.003*PaO_2$), which would mitigate distal villus ischemia, and c) as a cathartic, blood would tend to move through the bowel, both oxygenating and potentially eliminating deleterious substances from focal points of ischemia.

For reproductivity purposes maybe in the conclusions of the study you should highlight that the hemorrhagic shock was due to non-GI bleeding

This is now included in the Conclusion.

Reviewer 2:

… 1- The abstract needs to structured in sections (Aim, Methods, results, conclusion)

The abstract/paper is now formatted according to the instructions.

2- In line231 the author mentioned “These studies demonstrate that early increases in small bowel permeability occur during experimental hemorrhagic shock……” What is meant by these studies!! I think it is a typo as it should be written: This study

Now changed to read “This study”

3- At the end of discussion it would be better if the authors write a section titled Conclusions or a paragraph that starts with “in conclusion” summarizing the most important finding of the study

Last paragraph now begins with “In conclusion”

Reviewer 3:

Methods section: “Heparin was given (10 U/mL) to facilitate exsanguination…” Was heparin given systemically? Or was it used to wash the catheter lines/tubing to prevent clotting? If given systemically, this would alter the physiologic response to shock since the rats would be pre-treated and not exhibit the trauma-induced coagulation response following shock. Please clarify and note limitation.

Heparin was given systemically. This is now expressly noted in the Methods and is listed as a limitation. The Wiggers shock model relies on the slow exsanguination of animals to a set MAP. The MAP is maintained by withdrawing or replacing shed blood as needed to maintain the set MAP. Previous experience has shown that simply coating the catheter lines is not sufficient to maintain catheter patency and thus systemic heparinization is required. The Wiggers hemorrhagic shock model, while not very clinical, has the advantage of being the most reproducible of different hemorrhage models studied (e.g. withdrawal of set volumes, etc.). Systemic anticoagulation to some degree is a necessary condition for this model.

Was shed blood heparinized ex vivo as well? (before it was re-infused?)
Blood was not further heparinized

At what rate was the shed blood reinfused back? Was this a bolus injection or gradual transfusion using a syringe pump? Was any additional resuscitative fluid given to restore blood pressure? If so, please include this in your methods.
Re-infusion was done gradually, in 1 ml aliquots analogous to withdrawal of shed blood, in order to avoid acute hypervolemia that might trigger cardiac failure and/or volume-induced pulmonary edema. No other resuscitative fluid was given to restore blood pressure. This is now made apparent in the text.

The authors state that they collected blood samples every 20 minutes and measured fluorescence, but were any other additional tests performed?
All tests performed are as listed in the text. No additional sequential (e.g. every 20 minutes) tests were performed.

Were blood gases analyzed?
No. While the results might be interesting, they were not the focus of the study.

It would be beneficial if the authors can provide confirmation of reduced oxygen and increased lactate (low pH) over the shock period. I'm not convinced that there is truly significant "ischemia" occurring within 20 minutes of hemorrhage, as stated in the discussion. I would imagine significant ischemia occurs at the end of shock (~1 hr post hemorrhage).
It is agreed that post-hemorrhage is where *systemic* pH and lactate, as indicators of malperfusion, would be expected to be most pronounced. *Local* (small bowel villi) changes in pH and lactate are more difficult to quantify and may or may not occur within 20 minutes of hemorrhage, especially during a low-perfusion state. It is important to note is that there are local small bowel changes as measured by histology, immunohistochemistry, *in situ* zymography, and that these changes are also found to occur very rapidly in different forms of low-perfusion states to the bowel (Ref 1,2). Our data corroborate these findings.

Did the authors quantify any gut enzymes or other peptides from the acquired blood samples?
No. Gut enzymes are referenced in Refs 3, 30, 31.

In the methods, the author's state that "the heart, liver and lungs were collected, homogenized and quantified for fluorescence". Presenting this data in a table would be better. In fact I think a summary table with the most important findings could improve this paper. However, regarding the organ data, the variability (sd) seems quite high. It appears that there could have been a difference between the SHAM and SHOCK groups, but the variance is too high. Is this because of the handling of tissues? Was the same amount of tissue used (in grams?)
The same amount of tissue was used in all measurements. These results were not highlighted because they did not add important additional information to the manuscript. A table was originally considered.

The author's also state that there were significant baseline differences in the plasma and use this to justify normalization? Can the author's provide more explanation as to why there were baseline differences between the SHAM and SHOCK groups?
In as much as we used genetically identical rats of the same sex, age, and size, these models are difficult to ensure complete homogeneity in all aspects. There is some inherent variance in all animal models. Among these were baseline levels of casein fluorescence at time t=0.

This paper may benefit from having a Table 1 that summarizes the important findings from this study instead of having values embedded within the results section.
This suggestion is appreciated. The most pertinent findings, however, to the authors, are the time course of peptide egress into the systemic circulation and the visual demonstration of the fluorescent peptides in the microcirculation; neither lend themselves easily to tabular representation.

The statistical methods described don't seem to be appropriate for this study design. This is a repeated measures (longitudinal) study where samples were collected over time from a single animal. I would recommend at least using a repeated measures ANOVA than the student t-test. Longitudinal generalized estimating equations (GEE) or mixed models would be appropriate as well. I suggest consulting with a statistician to perform the appropriate tests.
We consulted with a statistician and he agreed that a more appropriate comparison test would be one of the above. A repeated measures ANOVA was conducted on the time-course data. Of note, there was a "new" lack of significance between groups at 160 minutes ($p=0.055$) but an apparent "new" significance between groups at 200 minutes. Measured significance between other groups did not appreciably change. Other paired/coupled statistical comparisons were kept as unpaired and paired (for the same sample before and after shock) two-tailed Student's t-tests.

While I understand and appreciate the use of FITC-dextran 20 for comparison to the FITC-casein peptide, I wonder if there is a confounding difference in the response to a protein-conjugated vs. starch-conjugated molecule? Why not use FITC-albumin?
The casein peptides are fluorescently conjugated, with some heterogeneity, to red-fluorescent BODIPY® TR-X. The FITC-dextrans 20 was used as a way to confirm the results achieved using the casein-conjugated peptides, as well as provide some measure of an upper bound of molecular weight (20kD); i.e., if 20kD dextrans was able to reach the systemic circulation then presumably peptides with smaller MW (leaving electrical charge out of the equation) would do so also, as the MW for (whole) casein is comparable. In comparison, the MW of albumin is approximately 65 kDa. Although a protein, it is nearly three times the size of intact casein, and thus not particularly useful as a marker for permeability, except to demonstrate the extent of bowel permeability to higher molecular weight substances. As mentioned in the Discussion, it would be of interest to conduct studies using molecular markers of defined molecular weights to determine permeability of different sized particles in time to a particular stimulus. In this way a series of curves could be constructed that correlate injury, length of ischemia/low flow state, with MW.

Figure 1 – I am a little confused as to why the fluorescent intensity (i.e. permeability) decreased over time in the SHAM group. Is this because of photobleaching of the dye? If so, was the rate of photobleaching accounted for in the SHOCK group?
Please see the response to similar questions from Reviewer #1. Both SHAM and SHOCK groups were treated in the same manner.

…I am a little confused as to why the images were not used for quantification (as a confirmatory step). It seems to me that there could have been ways of quantifying perfusion and flow rates

with this system to further validate the findings from the “serial blood draws”. Could the author’s please clarify and discuss.
This is a limitation to the study and is included in the Discussion on limitations.

Figure 3 – Did you observed infiltration of WBCs in the SHAM group? I would imagine that there is a level of inflammation just from the gut exteriorization and injection of dye in the bowel.
Not to our knowledge but we agree that this is certainly a possibility. This was not an objective of the study.

Figure 5 – I would show the same mesenteric location for “pre- and post-shock”. It currently looks like you picked a pre-ischemia image from a different spot than the post-reperfusion.
The images are from the same locations.

How does the mucosa layer compared to the endothelial glycocalyx layer? It appears that both layers play an important role in the protection of proteolytic and “autodigestion” activity.
The enteral mucus layer protects against the proteolytic (and probably bacterial) digestion of the mucosal (enterocyte) barrier. The glycocalyx is not well understood but may play a role in endothelial protection, as well as Starling forces, etc. There are some intriguing similarities between both layers.

Did you observe any accumulation of the FITC-labeled peptides in the lymphatic vessels?
We did not specifically observe the lymphatics and thus regrettably cannot comment. Deitch EA, et al, in a number of publications have consistently found the lymph to be a major conduit of inflammatory mediators from the bowel to the systemic circulation. Future studies will endeavor to visualize closer to the villus structures, including capillaries and primary lymphatics in real time.

Figure 4. – change the y-axis scale to (x103)
Changed

The authors use the terms “baseline”, “pre-ischemia”, “pre-shock” and “before hypotension” interchangeably throughout the text and figure captions. I would recommend choosing one term and keeping it consistent to avoid confusion to the lay reader.
Changed to “pre-ischemia” or “pre-ischemic”.

Thank you,

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