Author's response to reviews

Title: Effects of a Cholesterol-Enriched Diet on Intestinal Smooth Muscle Contraction: Inhibition of Muscarinic Receptors and their Disinhibition by the 5-HT4 Agonist - Tegaserod

Authors:

Ronald D Mathison (Ronald.Mathison@ucalgary.ca)
Eldon A Shaffer (shaffer@ucalgary.ca)

Version: 3 Date: 21 December 2005

Author's response to reviews: see over
Dear Editors:

Please find attached a revised manuscript entitled “Increased Cholinergic Contractions of Jejunal Smooth Muscle Caused by a High Cholesterol Diet is Prevented by the 5-HT4 Agonist – Tegaserod” by Ronald Mathison and Eldon Shaffer that we are re-submitting to BMC Gastroenterology for consideration towards publication.

Taking into consideration the referee’s comments the title of the manuscript has been changed from the original title “Effects of a Cholesterol-Enriched Diet on Intestinal Smooth Muscle Contraction: Inhibition of Muscarinic Receptors and their Disinhibition by the 5-HT4 Agonist – Tegaserod”.

We would like to thank the referees for their invaluable and insightful review of our manuscript. The comments and criticisms have certainly improved the focus of the manuscript, and help clear up some ambiguities. Replies to the referees are included in the attached files:
   1) Reply - Reviewer David Schneider.pdf

All authors have read and agreed to the content of this manuscript. The experimental research was performed with the approval of The University of Calgary Animal Care Committee, which conforms to the guidelines of the Canadian Council on Animal Care.
Sincerely,

Ronald Mathison (PhD)  

Eldon Shaffer (MD FRCPC)

**Contact Information**

Dr. Ronald Mathison  
Department of Physiology and Biophysics  
University of Calgary  
Calgary, AB, Canada T2N 4N1  
Telephone: 403-220-6896  
Fax: 403-283-4740  
Email: Ronald.Mathison@ucalgary.ca
Reviewer's report

Title: Effects of a Cholesterol-Enriched Diet on Intestinal Smooth Muscle Contraction: Inhibition of Muscarinic Receptors and their Disinhibition by the 5-HT4 Agonist – Tegaserod

Version: 1

Date: 12 October 2005

Reviewer: David Schneider

Reviewers report:

General

This is a new and well conducted study that demonstrates enhanced cholinergic contraction of the jejunum of ground squirrels on a high cholesterol diet. The result is somewhat surprising in that it has been previously shown that the muscarinic contraction of gallbladder smooth muscle is reduced in this model. The present study shows that enhanced jejunal contraction is due to loss of a competing muscarinic-induced nerve-mediated inhibition of jejunal smooth muscle. Some of the evidence presented suggests that the mechanism is dependent upon development of increased biliary cholesterol, at least within the hepatic duct. The results also uncover a novel effect of muscarinic stimulation in producing nerve-mediated smooth muscle inhibition in the jejunum, an effect not seen in the ileum. It is unfortunate that the effects of tegaserod on the distal small intestine were not also studied, as the enhanced peristalsis expected from tegaserod might have also affected the ileal exposure to luminal bile and increased cholesterol. The present findings, nonetheless, are new and informative.

------------------------------------------------------------------------

Significant Changes:

1) Title of manuscript has been modified to reflect the revised interpretation of the data, as recommended by the referee in point #3. The new title is: “Increased Cholinergic Contractions of Jejunal Smooth Muscle Caused by a High Cholesterol Diet is prevented by the 5-HT4 Agonist - Tegaserod”.

2) A new figure has been added. Figure 4 now shows “for future reference and potential comparison” data on ileal contractions – referees’ point #23(d).

------------------------------------------------------------------------

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

1. Statements need to clarify whether or not any change in baseline tone or spontaneous activity was observed in the presence of TTX, hexamethonium and scopolamine and, if so, how that was handled in data interpretation.
A new section was added to the results “Phasic and Tonic Contractions of Intestinal Segments” (page 9) addressing changes in baseline tone and spontaneous activity.

2. This article does not establish whether chronic (>28d) exposure was necessary to observe the effects of high cholesterol diet. Is there another reference that provides similar information for acute exposure in ground squirrels? If not, please indicate that the necessary time to develop enhanced jejunal contraction is not known and could have been an acute effect.

The statement “the time course for the modification of cholinergic receptor function by the cholesterol diet is not known and acute effects cannot be excluded.” has been added on page 14, lines 6-8.

3. This study did not distinguish between muscarinic receptor transduction mechanisms (i.e., inhibitory versus excitatory) (See first line of page 13). Strictly speaking, this study demonstrates a unique jejunal smooth muscle inhibitory mechanism that is dependent upon stimulation of an unknown type of muscarinic receptor and the activation of VGSCs. Please correct any wording that implies the observed mechanism necessarily results from stimulation of muscarinic receptors that are directly coupled to inhibitory transduction mechanisms (e.g., M2 receptors).

The wording that “implies that the observed mechanism necessarily results from stimulation of muscarinic receptors that are directly couples to inhibitory transduction mechanisms” has been removed from the manuscript.

4. With regard to the last sentence in middle paragraph on page 14: Pre-treatments with tegaserod are not equivalent to placing tegaserod in the tissue bath as in the referenced experiments (44-48, 51). Tegaserod certainly washes out in the tissue bath unless it is re-added. Tegaserod effects in your experiment must be prior to in vitro testing. Please correct this statement and reconsider tegaserod conclusions.

The comparison of tegaserod’s actions, as investigated in this study, to the responses of isolated intestinal tissues in an organ to serotonin has been removed. The conclusions with regards to tegaserod’s effects have been modified to reflect an undefined mechanism of action, possibly related to modification of cholesterol metabolites in the intestine.

5. The first sentence of the last paragraph on page 14 is potentially misleading. High cholesterol diet appears to have dis inhibited muscarinic contraction of jejunal smooth muscle similar to the effects of TTX. Since tegaserod reduced hepatic duct cholesterol, however, the effect of tegaserod may have been normalization of cholesterol exposure despite a continued high cholesterol diet. Note: Even though you found no statistical effect of tegaserod on gallbladder cholesterol in animals receiving high cholesterol diet, you have not tested whether or not gallbladder cholesterol remained elevated above baseline – i.e., above gallbladder cholesterol in non-treated animals on a low cholesterol diet. It appears unlikely that it remains significantly increased in tegaserod treated animals on high cholesterol diets. Therefore, instead of a reversal of the dis inhibition, it seems as likely that tegaserod may have prevented the increased cholesterol necessary
to produce the effect. Please consider these comments and reconsider tegaserod
conclusions.

We reanalyzed some bile samples and added additional data that were not
available at the time the original manuscript was prepared. As a result the
N-value for the bile composition studies was increased by 6 to 7. The
results remain unchanged.

6. The authors need to discuss the disparate effects of high cholesterol diet on
gallbladder versus jejunal versus ileal muscarinic contraction.

This issue is discussed on page 13, lines 4-15.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term,
which the author can be trusted to correct)

7. Methods; Experimental Animals: Considering these were wild animals used for
experimentation within a month of capture, can you state anything about the uniform
health of these animals, especially as relates to internal parasitism? Were animals given
an anthelmintic and, if so, what?

A comment on general health included on page 6, lines 5-6. An antihelmintic was
used - see page 6, lines 1 & 2.

8. Methods; Experimental Animals, Page 6: “Animals randomly divided into four groups
(19 in each)...”; so, this accounts for 76 of the 96 animals used. Please explain how the
other 20 were used or make corrections as necessary. The very next paragraph does
not explain which animals were used for the determination of hepatic and gallbladder
bile cholesterol concentration? Please clarify.

64 animals were used for this study. The 32 other animals indicated in the original
manuscript were part of another drug study, and the data were not used in the
current study. Clarification is provided on the use of animals, page 6, 1st
paragraph, last 3 lines.

9. Cholesterol Concentrations in Hepatic and Gallbladder Bile, page 6: Please define
“acute” in “acute terminal experiments in vivo...” Were these animals in addition to those
used in the contractility experiments? Please clarify.

Acute removed and sentence reworded – page 6, last 2 lines.

10. First line of page 7: remove extraneous “(“.

Removed.

11. First sentence under Intestinal Contractility in vitro, page 7: (a) Please clarify. Were
experiments started after 4 weeks on diet by testing 2 animals per diet group daily? (b)
Please clarify how preparations from each animal were used. Was it one jejunum/one
ileum for carbachol alone, one jejunum/one ileum for carbachol in the presence of TTX,
one jejenum for carbachol in the presence of hexamethonium, leaving one jejenum for carbachol in the presence of scopolamine? In the results (page 10), however, it is stated that ileal contraction was not modified by hexamethonium. In which animals were the effects of hexamethonium studied on ileal segments?

(a) Two animals were tested daily – page 6, 1st paragraph, last 3 lines. (b) Clarification on tissue is provided on page 8, lines 9-13.

12. Intestinal contractility in vitro, page 7: (a) Please state temperature of tissue bath system. (b) Please clarify if bethanechol was added cumulatively or non-cumulatively? If the latter, in what order were concentrations added? (c) Please state how long antagonists were allowed to pre-incubate before constructing concentration-response curves?

(a) Temperature: 35°C (page 7, line 10 of section “Intestinal Contractility in vitro”). (b) Carbachol was added cumulatively (page 8, lines 3-4). (c) Pre-incubation with antagonists: 10 min (page 8, lines 9-11).

13. Intestinal contractility in vitro, page 7: 0.1 µM TTX is pretty minimal; 0.3 µM is generally needed in guinea pigs and at least 1 µM in mice and rats to reliably block nerve-mediated effects. Is there any reference that documents this concentration is sufficient to block nerve-mediated activity (MMC, peristalsis, or electric-field induced)? Nonetheless, you observed an effect that might best be qualified as highly sensitive to block by TTX.

Use of 0.1µM TTX. Although TTX is commonly used at 1µM, there are data showing that 0.1µM TTX is effective in blocking neurogenic actions in intestinal preparations. Some citations are: Venkova K et al., Peptides. 1992; 13:193-201 (cat large intestine); Biagi B et al., Am J Physiol. 1990; 258:G223-30 (rabbit colon). A dose of 0.2µM TTX was effective in rat colon (Christofi FL et al., J Comp Neurol. 2004; 469:16-36, and 0.15µM TTX was used with mouse distal colon (Fontaine J, Lebrun P., Br J Pharmacol. 1989; 96:583-90).


15. Page 8: Please fix subject verb agreement in second line: “…, the tissue preparation were…”. Please consider revising second sentence of Data Analysis.

Corrected.

16. Data Analysis, page 8: Consider revising second sentence. Also, it should not be a mystery as to which statistical tests were used for each experiment. If not listed here, then it should be made clear in the Results or in the figure legends. [In my opinion, the cholesterol data should be tested with a two-way analysis of variance. The same could be done for predicted maximum effects for concentration-response curves. Addressing this additional comment is not essential as I don’t believe reanalysis would result in a
change in the statistical differences found and not found. Power may have been a problem for the cholesterol measurements in the gallbladder.]

**Data analysis: Clarification on the use of statistics (page 8, last paragraph).** A two-way analysis of variance was not used since the number of animals in each group was not the same.

17. Cholinergic Contractions of Jejunal Longitudinal Smooth Muscle, page 9: (a) Correct first sentence structure “…carbachol with increasing in tonic contractions…” and delete “optimal” as there is no indication as to why it is optimal; perhaps supramaximal. (b) Correct first sentence, second paragraph “…Newtons/m2…”

**Corrected.**

18. Effect of Action Potential Blockade on Carbachol-Evoked Contractions, page 9: (a) “Following TTX exposure …” is not correct. It was “In the presence of TTX …” (b) Second paragraph page 10: First sentence is awkward. Is the point that the TTX-induced enhancement of cholinergic jejunal contractions was not altered by pre-treatment with tegaserod in the low-cholesterol diet group? (c) Please make clear whether or not the effects of hexamethonium and scopolamine were tested on animals treated with tegaserod in the jejunum and ileum.

(a) Corrected. (b) The sentence has been reorganized to more clearly convey the results shown in Figure 2C (page 11, 2nd paragraph, lines 1&2). (c) Clarification added on page 8, lines 11-13.

19. Interactions…, page 11: Please correct text since you DO show the hexamethonium data in high cholesterol diet animals (Figure 3b).

**Corrected.**

20. References are expected for line one of page 12: “…influences the affinity, binding capacity and signal transduction.” Especially references related to such effects on muscarinic receptors.

**Reference [28] added - page 13, last paragraph, line 4.**

21. Bottom of page 13: (a) Reference 41 only shows increased cholesterol content in gallbladder smooth muscle, which has reduced (not enhanced) contractions. It may be too much to assume that jejunal smooth muscle or jejunal myenteric neuron membranes also have increased cholesterol content. Direct exposure of myenteric motor neurons to lumen and absorbed substances does not seem likely. Note also that exposure to plasma cholesterol (previously shown to be elevated in these animals) would likely be similar for myenteric neurons of jejunum and ileum. (b) High cholesterol diet reduced an opposing inhibitory mechanism to the smooth muscle that is mediated by activation of a muscarinic receptor and involves opening of TTX-sensitive VGSCs. Might the effects of high cholesterol diet on this inhibitory mechanism be due to loss of receptors, loss of receptor transduction mechanism, and/or loss of inhibitory mechanism?
(a) See new discussion - page 13, lines 1-4. (b) The reviewer points out a variety of mechanisms that the high cholesterol diet could exert an inhibitory effect. We do not have data that addresses these mechanisms so they have not been discussed.

22. Please clarify last sentence of page 13 that continues on to page 14.

This sentence has been deleted. A modified discussion on this topic is presented on page 13, 1st paragraph.

23. Figures (a) X-axis in all figures should be “log[Carbachol](M)” and not “[Carbachol].M”. (b) Correct supra-scripted end-bracket in Y-axis labels in all figures. (c) Figure 2: correct the spelling of tegaserod. (d) For future reference and potential comparison, you must present more detail of the data on ileal contractions. This might be easiest to achieve by inclusion of the concentration-response relationship figure.

(a) Corrected. (b) Corrected. (c) Corrected. (d) Concentration-response curves for the ileum have been included in a new Figure 4.

Discretionary Revisions (which the author can choose to ignore)

24. On page 2 within the Background of the Abstract: it is probably sufficient to use “5-HT4 receptor” instead of “5-HT4 serotonin receptor”. The latter is redundant and not used anywhere else in the manuscript.

Corrected.

25. For clarity throughout the manuscript, consider consistent referral to the diets as low versus high cholesterol with definition given in the Methods. Within the abstract, for instance, the diets are referred to as low, 1% or enriched, and in the Results as “normal”.

The revised manuscript only contains the terms “low cholesterol diet” and “high cholesterol diet” as defined in the methods page 6, lines 6-9.

26. Eighth line page 5: It is not clear why “drugs used to treat this condition should have minimal impact on gallbladder contractility.” Would a drug that increased biliary emptying not be potentially helpful in decreasing the likelihood of stone formation? In either case, your data show a significant reduction in hepatic duct cholesterol with chronic tegaserod. Is that to be viewed as unfavorable?

Wording has been changed from “have minimal impact on gallbladder contractility” to “not adversely affect gallbladder contractility.” – page 5, lines 8-9.

27. Regarding the normalization method: Although only a minor concern, are the authors certain that normalization using this formula remains appropriate after high cholesterol diet? In other words, is it known that the relationship of longitudinal smooth muscle area to whole preparation weight and length remains unchanged with diet? For instance, some diets reduce or increase intestinal length. While normalization of data is frequently helpful, do the basic relationships hold up if the data is not normalized in this way?
The data remains unchanged with the normalization procedure. The principle variable in this normalization procedure is tissue weight.

28. In my opinion, there is no need to repeatedly state “significantly… (P<0.05)” throughout the manuscript when you’ve already defined significance as P<0.05 in the Methods. The exception would be for figure legends, which should be able to be read alone.

Removed as suggested.

29. It might be worthwhile noting in the discussion that these animals are expected to be hypercholesterolemic such that a difference between jejunum and ileum may have been luminal exposure to increased cholesterol or bile salts but probably not in exposure to plasma cholesterol.

“Luminal exposure” to cholesterol and bile salts is indicated in the discussion – page 13, 1st paragraph.

30. Figures 1 and 2 show the same data, but in a different combination. In my opinion, Figure 1 is not necessary.

Even though Figures 1 and 2 show the same data, but in a different combination, the authors view this duplication as important for an appreciation of the effects of two variables – cholesterol (Figure 1) and tetrodotoxin (Figure 2). For example, the data shown in Figure 1A is repeated in Figure 2A and Figure 2B, and it is not intuitive to cross compare these two Figures to arrive at Figure 1.

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

Level of interest: An article whose findings are important to those with closely related research interests.

Quality of written English: Needs some language corrections before being published

Statistical review: No

Declaration of competing interests: I declare that I have no competing interests.
Reviewer's report

Title: Effects of a Cholesterol-Enriched Diet on Intestinal Smooth Muscle Contraction: Inhibition of Muscarinic Receptors and their Disinhibition by the 5-HT4 Agonist – Tegaserod

Version: 1

Date: 11 November 2005

Reviewer: David Q Wang

Reviewer's report:

General

---

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached) *(see below - What next?)*

---

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

---

Discretionary Revisions (which the author can choose to ignore)

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions.

The authors have replied to the major compulsory revisions that were raised by David Schneider, the other reviewer. For completeness these are included here.

1. Statements need to clarify whether or not any change in baseline tone or spontaneous activity was observed in the presence of TTX, hexamethonium and scopolamine and, if so, how that was handled in data interpretation.

   A new section was added to the results “Phasic and Tonic Contractions of Intestinal Segments” (page 9) addressing changes in baseline tone and spontaneous activity.

2. This article does not establish whether chronic (>28d) exposure was necessary to observe the effects of high cholesterol diet. Is there another reference that provides similar information for acute exposure in ground squirrels? If not, please indicate that the necessary time to develop enhanced jejunal contraction is not known and could have been an acute effect.
The statement “the time course for the modification of cholinergic receptor function by the cholesterol diet is not known and acute effects cannot be excluded.” has been added on page 14, lines 6-8.

3. This study did not distinguish between muscarinic receptor transduction mechanisms (i.e., inhibitory versus excitatory) (See first line of page 13). Strictly speaking, this study demonstrates a unique jejunal smooth muscle inhibitory mechanism that is dependent upon stimulation of an unknown type of muscarinic receptor and the activation of VGSCs. Please correct any wording that implies the observed mechanism necessarily results from stimulation of muscarinic receptors that are directly coupled to inhibitory transduction mechanisms (e.g., M2 receptors).

The wording that “implies that the observed mechanism necessarily results from stimulation of muscarinic receptors that are directly couples to inhibitory transduction mechanisms” has been removed from the manuscript.

4. With regard to the last sentence in middle paragraph on page 14: Pre-treatments with tegaserod are not equivalent to placing tegaserod in the tissue bath as in the referenced experiments (44-48, 51). Tegaserod certainly washes out in the tissue bath unless it is re-added. Tegaserod effects in your experiment must be prior to in vitro testing. Please correct this statement and reconsider tegaserod conclusions.

The comparison of tegaserod’s actions, as investigated in this study, to the responses of isolated intestinal tissues in an organ to serotonin has been removed. The conclusions with regards to tegaserod’s effects have been modified to reflect an undefined mechanism of action, possibly related to modification of cholesterol metabolites in the intestine.

5. The first sentence of the last paragraph on page 14 is potentialy misleading. High cholesterol diet appears to have disinhibited muscarinic contraction of jejunal smooth muscle similar to the effects of TTX. Since tegaserod reduced hepatic duct cholesterol, however, the effect of tegaserod may have been normalization of cholesterol exposure despite a continued high cholesterol diet. Note: Even though you found no statistical effect of tegaserod on gallbladder cholesterol in animals receiving high cholesterol diet, you have not tested whether or not gallbladder cholesterol remained elevated above baseline – i.e., above gallbladder cholesterol in non-treated animals on a low cholesterol diet. It appears unlikely that it remains significantly increased in tegaserod treated animals on high cholesterol diets. Therefore, instead of a reversal of the disinhibition, it seems as likely that tegaserod may have prevented the increased cholesterol necessary to produce the effect. Please consider these comments and reconsider tegaserod conclusions.

We reanalyzed some bile samples and added additional data that were not available at the time the original manuscript was prepared. As a result the N-value for the bile composition studies was increased by 6 to 7. The results remain unchanged.
6. The authors need to discuss the disparate effects of high cholesterol diet on
gallbladder versus jejunal versus ileal muscarinic contraction.

This issue is discussed on page 13, lines 4-15.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No

Declaration of competing interests:
I declare that I have no competing interests

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

Level of interest: An article whose findings are important to those with closely related research interests.

Quality of written English: Needs some language corrections before being published

Statistical review: No

Declaration of competing interests:
I declare that I have no competing interests.