ANSWERING REVIEWERS

April 15, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 2248-review.doc).

Title: PHYTOESTROGENS/INSOLUBLE FIBERS AND COLONIC ESTROGEN RECEPTOR BETA: RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED STUDY

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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 reviewers:

Review (1)

- This is an interesting report and while a number of the findings are near-significant rather than being significant, in my view the study should be published and hopefully encourage further, perhaps larger, studies to be undertaken.
  Although the data is summarised in a reasonable manner, I would have liked to have seen a little more of the initial data to better gauge variation and spread of the values.
  We agree that the manuscript would have been enriched by the data of the period prior to treatment with phytoestrogens. However, it was impossible to get them, because the Ethics Committee has allowed us to perform this study with a single colonoscopy (after diet) that was appropriate as a follow-up investigation in patients with a history of polypectomy. Therefore, it was possible to obtain only data concerning diet versus placebo.

- For the IHC data, was it simply the percentage of cells positive that was evaluated or was there any attempt to look at intensity / strength of staining also?
  According to various reports in the literature and with the suggestion of the reviewer, we have re-evaluated the data based on the intensity of immunohistochemistry staining divided into weak, moderate and strong. This assessment did not change the results of our labelling index, as the percentage of weakly stained cells was less than 10%. Additionally, our results had already been referred only to moderate-strong intensity staining.

- Minor changes to the english are needed in a few places.
  Linguistic revision was performed by an expert native speaker (original letter enclosed).

Review (2)

- Mariabeatrice P. et al. reported in the paper entitled, "Dietary phytoestrogens and insoluble fibers increase estrogen receptor beta expression in the colon mucosa of patients with colonic adenomas. A randomized, double-blind, placebo-controlled study," that ER beta protein was upregulated in the colonic biopsies and this receptor was co-localized with caspase-3, leading to the conclusion that ER beta was involved in apoptosis. The idea about using dietary supplements to suppress ER beta-mediated colon tumorigenesis is very interesting. However, these authors failed to provide convincing evidence to support that their dietary supplements make any difference and ER beta plays any role in tumorigenesis.

  The aim of our study could not be to demonstrate that dietary supplementation had a chemopreventive effect on the growth of intestinal polyps due to the long time development of these lesions (years) as well as the short duration of the diet (two months). The real goal has been, therefore, the assessment of some biomarkers (estrogen receptors and indicators of proliferation / apoptosis) after a short period of diet. Obviously, highly significant results were not in our expectations, although a trend of our dietary supplementation to modify the biomarkers is clear from our study. Furthermore, study on the chemopreventive effect of our diet have been either studied (reference 20) and in progress in animal models. Concerning the ER beta role in colonic tumorigenesis, there is a large number of evidences in the literature (a very fitting example could be: Kennelly et al, Lancet Oncol 2008; 9: 110-91).

- Some specific comments are as follows:

  1. Grammatical error on last sentence on p5: "Finally, there is evidence…bind to an activate (?) ER with (?) chemopreventive effects…"

  The sentence was completely rephrased; its meaning was to report literature findings about the beneficial effect of some herbs on colon pre-cancerous lesions through an interaction with apoptosis.

  2. Bullet points at the end of introduction (p6) should be revised into sentences.

  No bullet point was reported and the end of the introduction was rephrased into simple sentences clearly focusing the aim of the work.

  3. p14: Authors claimed that "none showed high grade dysplasia." What is the significance of slight increase of the ER beta protein then? Authors should elaborate.

  As reported in the first answer, the real goal of our study has been the assessment of some biomarkers (estrogen receptors and indicators of proliferation / apoptosis) after a short period of diet with phytoestrogens and enterolignans. A short period of diet may not affect the polyp natural history or dysplasia degree.

  4. p15 and Table 2: Authors reported that the increase of the ER beta protein was statistically significant. However, the difference was only marginal with P=0.04, which only very weakly justified that the observed difference is indeed real/interesting. Additional data to support this increase would be necessary. For example, any data to show that this increase in ER beta has functional implication would be good (any increase in target gene expression for example). The fact that ER beta message levels were no difference, suggesting that change of gene transcription and mRNA stability do not explain the increase of the receptor protein and protein degradation might play a role in the difference of the ER beta protein. Authors should elaborate about the mechanisms involved in the increase of the ER beta protein with cited

literature or data.

In our study, we found that ADI induced a statistically significant increase of ER-beta protein, whilst ER-beta mRNA levels were higher than in placebo group but the difference did not reach significance. Moreover, both the protein and mRNA were significantly increased in the subjects without the finding of polyps at endoscopy. A possible explanation of our result may be due to an increased synthesis as well as a reduced degradation of ER-beta protein. Indeed, the transcription, synthesis and degradation of ER protein are processes subjected to mechanisms of complex control through highly regulated adjustment systems. Degradation, in particular, takes place in the cell by a cytosolic complex, the proteasome. It is mediated by ubiquitin that binds to the ligand binding domain of the receptor, i. e. ubiquitination (Nawaz Z et al, Biochemistry 1999; 96: 1858-62, Reid et al, Cell Mol Life Sci, 2002; 59: 821-31). It is possible that these mechanisms may be different in the colonic mucosa predisposed or not to development of polyps. Finally, this is a preliminary report and a complete study about target gene expression is enclosed in our future investigation projects.

5. First sentence under "Treatment-related immunohistochemical biomarkers" – "The median value…" – does not make sense and should not be an one-sentence paragraph.

As suggested, the sentence was deleted in revised manuscript.

6. p18: Authors stated that "…correlation tests showed that ER-beta was directly linked to apoptosis." This statement is too strong and authors did not show convincing data to make this conclusion. The co-expression immunohistochemical data per se are not adequate. Some in-vitro (cell culture) data should be helpful to provide useful data.

The statement was changed. Indeed, we are conscious that our finding of immunohistochemical co-expression of ER-beta and caspase 3 does not constitute an irrefutable proof of a direct link. However the literature clearly show that ER-beta can upregulate proteins p21 and p27, which in turn activate the caspase 3 and 8 to induce apoptosis in Lo Vo cells, i. e. transient transfected cellular elements used with the aim of evaluating a relationship between estrogen receptors and apoptosis (Hsu HH et al, Chin J Physiol 2006; 49: 100-116).

7. p19 and Table I: Authors stated that "…ADI substantially increased phytoestrogen levels…" which actually weakened the argument that ADI upregulated the ER beta protein. It appeared that ADI was not that effective at all to upregulate the receptor.

Table 1 shows the urinary levels of enterolignans at time 0 and after 30 and 60 days. The assay was conducted to demonstrate the adherence to the diet of the subjects as well as to avoid that external dietary factors could affect our results. This evaluation could be an indirect index of phytoestrogen level increase in ADI group. On the other hand, literature evidences showed that a high estrous cycle duration, increased corticosterone and 17beta-estradiol levels with an overexpression of receptors ER-alpha and ER-beta in animal model (Amorim JP et al, Reprod Biol Endocrinol. 2011; 9:160).

Review 3

- This manuscript is well organized and interesting. I have one question in results. The author described that "In recurrent patients a higher ER-beta protein (p=0.04) and a lower ER-alpha LI (p=0.02), in ADI group, was also disclosed." This phenomenon was seemed to be controversial against the role of ER-beta in this manuscript. Could you explain this matter?

  The apparent contradiction of these results can be explained by the fact that the aim of our study could not be to demonstrate that our dietary supplementation had a chemopreventive effect on the growth of intestinal polyps due to the long time development of these lesions

(years) as well as the short duration of the diet (two months). The real goal has been the assessment of some biomarkers (estrogen receptors and indicators of proliferation / apoptosis) after a short period of diet. Additionally, the estimation was performed in normal mucosa of recurrent and non recurrent patients.

3 References and typesetting were corrected

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

Sincerely yours,

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