Format for ANSWERING REVIEWERS

May 21, 2015

Dear Editor and Reviewers,

We much appreciate the valuable comments provided by the Editor and Reviewers. We have revised our manuscript based on your recommendations; the revisions are indicated in red font and highlighted. Please find the edited manuscript in Word format (file name: 17125-review.doc). We have also provided a summary of the changes in response to each comment below.

Thinking that these revisions have properly improved the manuscript, we believe it is now acceptable for publication. Would you please let us know at your earliest convenience if there are any problems that still need our attention?.

Title: Broccoli sprout extract induces detoxification-related gene expression and attenuates acute liver injury

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Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 17125

The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

(1) We added the following statements related to academic rules and norms in the title page.

Institutional Review Board (IRB) statement:

This study is not human study, so there is no Institutional Review Board (IRB) statement to disclose.

Institutional Animal Care and Use Committee (IACUC) statement:

The animal experiment protocol was approved by the Committee on the Care and Use of Laboratory Animals of the Kagome Animal Use Committee (2007.005, 2007.016, and 2008.011).

Animal care and use statement:

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were housed at 20–24°C and 45–65% humidity in an animal laboratory with a 12-h light/12-h dark cycle timed from 7:00 AM. They were fed a normal commercial diet and sterile water during the 5-day acclimatization period before the experiment. APAP administration via p.o. was carried out with conscious animals, using flexible plastic needles appropriate for the animal size (200 g body weight: 110 mm length, 1.6 mm ball diameter). D-GalN administration via i.p. was carried out with conscious animals, using disposable injection needles appropriate for the animal size (200 g body weight: 25 gauge, 16 mm length, 0.50 mm diameter).All animals were euthanized by pentobarbital sodium (i.p., 50 mg/kg) for tissue collection.

Conflict-of-interest statement:

No potential COI to disclose.

Data sharing statement:
Technical appendix, statistical code, and dataset available from the corresponding author at "Kazutaka_Yoshida@kagome.co.jp".

(2) We added the title of the corresponding author.

(3) We prepared the Audio Core Tip and submitted it.

(4) We corrected the format of all Tables (1-9) to Excel format and all Tables were put at the end of this paper.

(5) COMMENTS was added as follows (p. 19-20, lines 359-381)

Background
Broccoli sprout is a unique plant which is abundant in sulforaphane, known as the most potent naturally occurring inducer of phase II drug-metabolizing enzymes. However, few reports have evaluated the effects of continuous ingestion of broccoli sprouts on liver function. Thus, it was very important to clarify the effects of broccoli sprout intake on gene expression in liver.
Research frontiers
To our knowledge, there have been no reports showing a detailed analysis of daily administration of dietary broccoli sprouts on liver gene expression. In this study, we used DNA microarray to investigate the effects of broccoli sprout extract (BSEx) on gene expression in rat liver. That technology allowed us to comprehensively analyze the expression of a large number of genes in liver.
Innovations and breakthroughs
We showed that BSEx upregulated the expression of genes related to detoxification and glutathione synthesis in normal rat liver using DNA microarray and real-time PCR analyses. Moreover, BSEx suppressed APAP- and D-GalN-induced liver injury. We conclude that BSEx enhanced defensive functions and protected against the toxicities of various types of xenobiotic substances through induction of detoxification enzymes and glutathione synthesis in the liver.
Applications
Broccoli sprout is a commercially available plant and it is fit for food. The results of this study are applicable for development of functional foods using BSEx, although human trial will be needed in the future.
Peer review
It is an interesting study investigating the effect of BSEx on gene expression in rat liver, and on the intoxication produced by acetaminophen (APAP) and D-galactosamine (D-GalN). The results from these studies may provide better insights into the hepatoprotective effects of broccoli sprouts.

2 Revision has been made according to the suggestions of the reviewer

Answer to reviewer No. 02505493

(1) In a work published recently [Mol Nutr Food Res 2014; 58(10): 1991–2000] the authors have noted a decrease in weight gain in animal fed with broccoli extract – containing diet that might be resulted to the decreased food consumption due to the bitter flavor of broccoli extract or pure sulforaphane. In the present m/s, the authors show no difference (Table 8) and an increase (Table 9) of weight gain in the animals (control vs BSE), with no difference in food consumption. This controversy should be discussed.

Our response:

We think that the following two factors are related with the controversy indicated by the reviewer.

The first one is the extraction temperature. In the previous study, broccoli sprout extracts were prepared by 60°C mildly heating or 5-min steaming of broccoli sprouts. Under the condition of 60°C mildly heating, myrosinase has its enzyme activity and converts glucosinolates to isothiocyanates. Because of the bitter taste of isothiocyanates, rats might avoid eating broccoli extract - containing diet.

The second one is the germination stage of broccoli sprouts. We used 1-day old broccoli sprout for extraction of BSEx, but, in the previous study, 6-day old broccoli sprouts were used. It was reported that the content of phenolic compounds in broccoli sprouts increased dependent on its germination stage, so 6-day-old sprouts were thought to contain more phenolic compounds than 1-day broccoli sprouts. Because of the bitter taste of phenolic compounds, rats might avoid eating broccoli extract - containing diet.

Based on the above, we added following paragraph in DISCUSSION section (p.16, lines 265-278).

"In the previous study, mice consuming the broccoli sprout extract-containing diet gained significantly less weight than mice consuming the normal diets and the reason is thought that the bitter flavor imparted by the broccoli sprout extract may have resulted in decreased consumption compared to the normal diet[32]. However, we show no decrease (Table 8, 9) of weight gain in the animals fed with BSEx diet, with no difference in food consumption (data not shown). We think that the following two factors are related with the controversy. The first one is the extraction temperature. In the previous study, broccoli sprout extracts were prepared by 60°C mildly heating or 5-min steaming of broccoli sprouts[32]. Under the condition of 60°C mildly heating, myrosinase has its enzyme activity and converts glucosinolates to isothiocyanates. The second one is the germination stage of broccoli sprouts. We used 1-day old broccoli sprout for extraction of BSEx, but, in the previous study, 6-day old broccoli sprouts were used[32]. It was reported that the content of phenolic compounds in broccoli sprouts increased dependent on its germination stage, so 6-day-old sprouts were thought to contain more phenolic compounds than 1-day broccoli sprouts[33]. Because of the bitter taste of isothiocyatates and/or phenolic compounds, rats might avoid eating broccoli extract containing diet in the previous study."

Accordingly, we added the following references;

32. **Bricker GV**, Riedl KM, Ralston RA, Tober KL, Oberyszyn TM, Schwartz SJ. Isothiocyanate metabolism, distribution, and interconversion in mice following consumption of thermally processed broccoli sprouts or purified sulforaphane. *Mol Nutr Food Res* 2014; **58**: 1991-2000. [PMID: 24975513 DOI: 10.1002/mnfr.201400104]
33. **Waje CK**, Jun SY, Lee YK, Moon KD, Choi YH, Kwon JH. Seed viability and functional properties of broccoli sprouts during germination and postharvest storage as affected by irradiation of seeds. *J Food Sci* 2009; **74**: C370-374 [PMID: 19646029 DOI: 10.1111/j.1750-3841.2009.01161.x]

(2) the authors suggest that their findings support the daily consumption of broccoli sprout extract in order to protect the liver from various types of xenobiotic substances through induction of detoxification enzymes and glutathione synthesis. Actually, they don't use a brief extract, but an extract of boiled broccoli sprout, concentrated to contain high amounts of glucoraphanin. According to this, the authors should discuss their results with the results of other studies, not cited in the m/s, like that of Perocco et al. [Mutation Research 2006; 595 (1–2): 125–136] and that of Lai et al. [Food and chemical toxicology 2008; 46 (1): 195–202].

Our response:

According to the reviewer's indication, we added following paragraph to DISCUSSION section and 2 references to References section (p.16-17, lines 279-294).

"In this study, we prepared the BSEx diet containing 340 mg glucoraphanin/100 g diet. If calculated by the daily food intake (approximately 15 g) by a rat, and the body weight of a rat (approximately 200 g), we assumed that rats consumed about 200 – 300 mg/kg of glucoraphanin every day. Previous studies reported that 30–60 mg/kg of glucoraphanin administration was safe and effectively enhanced NQO1 (phase II enzyme) in various tissues and 120-240 mg/kg of glucoraphanin administration caused oxidative stress in rat liver[34, 35]. However, in this study, rats didn't show any of AST, ALT, TBARS increase and GSH decrease after 10 days of BSEx diet administration (Table 8, 9). These results suggested that BSEx used in this study had higher level of safety than used in the previous study. On the other hand, a pilot study (data not shown) carried out before this study showed that APAP-induced liver injury was strongly suppressed by the administration of BSEx diet containing 170 mg glucoraphanin/100 g diet (about 100 – 150 mg/kg of glucoraphanin every day). Moreover, APAP-induced liver injury was weakly suppressed by the administration of BSEx diet containing 34 mg glucoraphanin/100 g diet (about 20 – 30 mg/kg of glucoraphanin every day). These results suggest that acute liver injuries were suppressed by the low BSEx administration than in this study. We conducted experiment using high glucoraphanin contained diet to clarify the effect of BSEx on the liver injuries, but we think that the investigation of the dose-response effect of BSEx will be needed in future studies."

34. **Perocco P**, Bronzetti G, Canistro D, Valgimigli L, Sapone A, Affatato A, Pedulli GF, Pozzetti L, Broccoli M, Iori R, Barillari J, Sblendorio V, Legator MS, Paolini M, Abdel-Rahman SZ. Glucoraphanin, the bioprecursor of the widely extolled chemopreventive agent sulforaphane found in broccoli, induces phase-I xenobiotic metabolizing enzymes and increases free radical generation in rat liver. *Mutat Res* 2006; **595**: 125-136 [PMID: 16442570 DOI: 10.1016/j.mrfmmm.2005.11.007]
35. **Lai RH**, Keck AS, Wallig MA, West LG, Jeffery EH. Evaluation of the safety and bioactivity of purified and semi-purified glucoraphanin. *Food Chem Toxicol* 2008; **46**: 195-202 [PMID: 17804139 DOI: 10.1016/j.fct.2007.07.015]

(3) Do not use the abbreviation BSE, since it is used for Bovine spongiform encephalopathy.

Our response:
The abbreviations "BSE" used in our manuscript and Tables (1, 7, 8, 9) were all changed to the abbreviation "BSEx".

Answer to reviewer 573188
The comments by reviewer No. 573188 were unfinished because of the system error.
If there are other comments by reviewer No. 573188, please provide them for next revision.

(1) Precisely, the first part of the document is devoted to expression changes, a parameter that is not explored in the two animal models used in the second part

Our response:
In the first part of this document, we conducted gene expression analysis to clarify the effects of broccoli sprouts on the gene expression in rat liver. We think that "expression change" is generally used to evaluate the effects of food ingredients or drugs on gene expression. Therefore, we showed the results of the first part by using "expression change".
In the latter part of this document, we conducted the experiments using acute liver injury models to assess the effects of broccoli sprouts on the phenotype of rat liver. In general, "expression change" is not used to show the results of liver injury model. Therefore, we didn't use "expression change" to show results of the experiments using acute liver injury model.

(2) Additionally, the second part of the manuscript lacks histological data that are key to claim the beneficial effects of BSE in liver injury.

Our response:
We think that histological data are important to assess the effects of broccoli sprouts intake on acute liver injuries. However, we wanted to evaluate the effects of broccoli sprouts intake on rat liver from the basic perspective. Therefore, we evaluated the effects of broccoli sprouts on the rat liver injury by using only AST and ALT, those are widely used to evaluate liver injuries, and measured biochemical markers, gene expressions, enzyme activities, antioxidant activities, and GSH concentrations to particularly understand the effects of broccoli sprouts on the rat liver.

(3) Finally, a need to translate the dose of BSE used into broccoli servings will clarify the feasibility of preventing drug liver injury through the diet.

Our response:
The aim of this study is to clarify the effects of BSEx on gene expression in rat liver; therefore, the concentration of BSEx in the diet was adjusted to the concentration, which is expected to show a clear effect on rat liver based on pilot animal experiments using acute liver injury models. The concentration in BSEx diet was not based on the intake amount of broccoli sprouts by humans.
Calculated by the daily food intake (approximately 15 g) by a rat, rat intaked about 50mg of glucoraphanin every day. When the amount was converted only based on the weight of rat (approximately 200 g) and man (approximately 70 kg), a man of 70kg need to eat about 17.5 g of grucoraphanin. The concentration of glucoraphanin in the broccoli sprouts using in this study was 2 - 3 mg/g fresh weight. Therefore, 17.5 g of grucoraphanin was equivalent to approximately 6 - 9 kg of broccoli sprouts.
However, we think that simple comparisons of broccoli sprouts intakes between the rats and humans are problematic in this study because the differences in glucoraphanin assimilation rates between rats and humans are currently under investigation.

(4) Abstract 1- acetaminophen is commonly abbreviated as APAP.

Our response:
The abbreviations “AAP” used in our manuscript and Tables 8 were all changed to the abbreviation “APAP”.

(5) Abstract 2- The regime used for drug treatment is not clearly stated in the abstract. Precisely, when are the drugs administered and the via (i.p., s.c, etc.) through which they are provided should in my opinion be clearly stated.

Our response:
We added the phrase “APAP administration via p.o. or D-GalN administration via i.p” (p.4, line 12).

(6) Abstract 3- Data regarding AST and ALT should be separated to make the text more comprehensible.

Our response:
We changed the sentence as follows;
p.4, lines 18-20; ” The levels of AST (70.91 ± 15.74 IU/mL vs 5614.41 ± 1997.83 IU/mL, $p < 0.05$) and ALT (11.78 ± 2.08 IU/mL vs 1297.71 ± 447.33 IU/mL, $p < 0.05$) were significantly suppressed in the APAP+BSEx group compared with the APAP group.”
p.4, lines 23-25; ” AST (4820.05 ± 3094.93 IU/mL vs 12465.63 ± 3223.97 IU/mL, $p < 0.05$) and ALT (1808.95 ± 1014.04 IU/mL vs 3936.46 ± 777.52 IU/mL, $p < 0.05$) levels were significantly suppressed in the D-GalN+BSEx group compared with the D-GalN group.”

(7) Abstract 4- Although a significant reduction in AST and ALT values is observed by BSE administration in D-GalN intoxication, their levels are still far over normalcy, and far over those of BSE in APAP intoxication. Hence, I would suggest the authors to state this clearly already in the abstract.

Our response:
According to the reviewer's comment, we changed the sentences as follows;
Abstract p.4-5, lines 23-26; " AST (4820.05 ± 3094.93 IU/mL vs 12465.63 ± 3223.97 IU/mL, $p < 0.05$) and ALT (1808.95 ± 1014.04 IU/mL vs 3936.46 ± 777.52 IU/mL, $p < 0.05$) levels were significantly suppressed in the D-GalN+BSEx group compared with the D-GalN group, but the levels of AST and ALT in the D-GalN+BSEx group were higher than those in the APAP+BSEx group"
RESULTS p.15, lines 255-257; " Increases in AST and ALT in the D-GalN group were significantly suppressed in the D-GalN+BSEx group, but the levels of AST and ALT were much higher than that in the APAP+BSEx. Increases in TBARS was not significantly suppressed (Table 9)."

(8) Abstract 5- No mention of effects on GSH levels in the D-GalN model is made and vice versa no mention to effects on GST activity in APAP intoxication is described.

Our response:
We didn't measure the GSH levels in the D-GalN model because increases in TBARS level by D-GalN were not significantly suppressed by BSEx. Therefore, we didn't mention the effects on GSH levels in the D-GalN model.
Liver GST activity was significantly increased in the APAP+BSEx group compared with the APAP group. Based on this result, we changed the sentences as follows;
Abstract p.4, lines 20-23; "The level of GSH (2.61 ± 0.75 nmol/g tissue vs 1.66 ± 0.59 nmol/g tissue, $p < 0.05$) and liver GST activity (93.19 ± 16.55 U/g tissue vs 51.90 ± 16.85 U/g tissue, $p < 0.05$) were significantly increased in the APAP+BSEx group compared with the APAP group."

(9) Abstract 6- The conclusion in my opinion should be changed, since the authors supply the diet with a broccoli sprout extract not with broccoli sprouts.

Our response:
According to the reviewer's comment, we changed the sentences as follows;
Abstract p.5, lines 30-31; "We demonstrated that BSEx protected the liver from various types of xenobiotic substances through induction of detoxification enzymes and glutathione synthesis."
DISCUSSION p.19, lines 349-351; " We conclude that BSEx enhanced defensive functions and protected against the toxicities of various types of xenobiotic substances through induction of detoxification enzymes and glutathione synthesis in the liver."

(10) Introduction 1- lines 78-81. In my opinion, the effects investigated are not of "BSE on APAP and D-GalN", but rather on the intoxication produced by those drugs.

Our response:
We added the phrase "the intoxication produced by" (p.7, line 72)

(11) Materials and Methods 1- There is a typing error in the section title.

Our response:
We corrected the section title (p.8, line 75).

(12) Materials and Methods 2- As stated in the Introduction, "several day-old broccoli sprouts have 15-fold more glucoraphanin than mature plants". Hence, wouldn't it be better to use broccoli sprouts more than 1-day old for preparation of the extract? If there is any problem in using older sprouts could you please justify that in the text?

Our response:
It was reported that glucoraphanin content in broccoli sprout at the early stage of germination (within 24 h) was almost same as the glucoraphanin content at 72 h of germination. Therefore, we used 1-day old broccoli sprouts to conduct experiments in a short period.
We changed the sentences in the MATERIALS AND METHODS section as follows (p.8, lines 77-79).
"It was reported that glucoraphanin content in broccoli sprout at the early stage of germination (within 24 h) was almost same as the glucoraphanin content at 72 h of germination[17]. Therefore, BSEx was industrially processed using 1-day-old broccoli sprouts to conduct experiments in a short period."
Accordingly, we add the following reference;

17. **Gu Y**, Guo Q, Zhang L, Chen Z, Han Y, Gu Z. Physiological and biochemical metabolism of germinating broccoli seeds and sprouts. *J Agric Food Chem* 2012; **60**: 209-213 [PMID: 22142148 DOI: 10.1021/jf203599v]

(13) Materials and Methods 3- I would like to know the equivalence between the BSE dose administered and the amount of daily broccoli servings that a man of 70 kg would need to eat to reach that level of glucoraphanin. Is the equivalent dose reasonable or it means eating a very large amount of broccoli?

Our response:
The aim of this study is to clarify the effects of BSEx on gene expression in rat liver; therefore, the concentration of BSEx in the diet was adjusted to the concentration, which is expected to show a clear effect on rat liver based on pilot animal experiments using acute liver injury models. The concentration in BSEx diet was not based on the intake amount of broccoli sprouts by humans.
Calculated by the daily food intake (approximately 15 g) by a rat, rat intaked about 50mg of glucoraphanin every day. When the amount was converted only based on the weight of rat (approximately 200 g) and man (approximately 70 kg), a man of 70kg need to eat about 17.5 g of grucoraphanin. The concentration of glucoraphanin in the broccoli sprouts using in this study was 15mg/g fresh weight (data not shown). Therefore, 17.5 g of grucoraphanin was equivalent to approximately 1.2kg of broccoli sprouts.
However, we think that simple comparisons of broccoli sprouts intakes between the rats and humans are problematic in this study because the differences in glucoraphanin assimilation rates between rats and humans are currently under investigation.

(14) Materials and Methods 4- Table 1 states the diet composition, but it seems that knowledge about additional components of the BSE extract are lacking. These components (5.9%) include protein, and hence amino acids required for GSH synthesis. Have the authors any data concerning differences in these parameters that may require additional adjustments in the diet composition?

Our responses:
A quantitative description of the complete diet composition would be informative, but in this study, we did not clarify the components in BSEx except glucoraphanin. Inferred from general information about the composition of broccoli sprouts, most of the components in BSEx are protein or carbohydrate. But, their amounts are much fewer than those in AIN-76 diet. Also, we think that the amount of amino acids in BSEx is much fewer than that derived from casein in AIN-76 diet. Therefore, it was not likely that the other components than glucoraphanin or other

glucosinolates.

(15) Materials and Methods 5- lines 117-119 state that freezing of the livers was carried out just by storage at -80oC. The tissue was not flash frozen in liquid nitrogen before storage? Was the quality of the RNA not affected by a "slower" freezing process?

Our response:
Tissues were frozen in liquid nitrogen before storage, so we added the phrase "frozen in liquid nitrogen and" (p.9, line 103).

(16) Materials and Methods 6- lines 130-131. It seems that words are missing in this particular sentence.

Our response:
We added the word "hybridized" (p.9, line 113).

(17) Materials and Methods 7- Was the RNA used in real-time RT-PCR treated with DNase?

Our response:
The RNA used for real-time PCR was treated with DNase.
We added the phrase ", which is treated with DNase," (p.9, line 121).

(18) Materials and Methods 8- Greek letter are missing throughout the text.

Our response:
I'm sorry, but we couldn't find missed Greek letters. Could you tell me a concrete example?

(19) Materials and Methods 9- Table 2. Rat gene symbols should use the standard nomenclature capitalizing only the first letter. Concentrations of the primers used in real-time RT-PCR should be included.

Our response:
We changed rat gene symbols in Table 2 to capitalizing only the first letter.
Also, we changed rat gene symbols in MATERIALS AND METHODS, RESULTS, and DISCUSSION in the same way.
The concentration of each primer used in real-time PCR was 0.4 μM.
We added the sentence "The concentration of each primer used in real-time PCR was 0.4 μM." (p.10, lines 127-128).

3 References and typesetting were corrected

As stated in the answer to the reviewers, we added the following 5 references. And accordingly, reference numbers were corrected.

17. **Gu Y**, Guo Q, Zhang L, Chen Z, Han Y, Gu Z. Physiological and biochemical metabolism of germinating broccoli seeds and sprouts. *J Agric Food Chem* 2012; **60**: 209-213 [PMID: 22142148 DOI: 10.1021/jf203599v]
32. **Bricker GV**, Riedl KM, Ralston RA, Tober KL, Oberyszyn TM, Schwartz SJ. Isothiocyanate metabolism, distribution, and interconversion in mice following consumption of thermally processed broccoli sprouts or purified sulforaphane. *Mol Nutr Food Res* 2014; **58**: 1991-2000. [PMID: 24975513 DOI: 10.1002/mnfr.201400104]
33. **Waje CK**, Jun SY, Lee YK, Moon KD, Choi YH, Kwon JH. Seed viability and functional

properties of broccoli sprouts during germination and postharvest storage as affected by irradiation of seeds. *J Food Sci* 2009; **74**: C370-374 [PMID: 19646029 DOI: 10.1111/j.1750-3841.2009.01161.x]

34. **Perocco P**, Bronzetti G, Canistro D, Valgimigli L, Sapone A, Affatato A, Pedulli GF, Pozzetti L, Broccoli M, Iori R, Barillari J, Sblendorio V, Legator MS, Paolini M, Abdel-Rahman SZ. Glucoraphanin, the bioprecursor of the widely extolled chemopreventive agent sulforaphane found in broccoli, induces phase-I xenobiotic metabolizing enzymes and increases free radical generation in rat liver. *Mutat Res* 2006; **595**: 125-136 [PMID: 16442570 DOI: 10.1016/j.mrfmmm.2005.11.007]
35. **Lai RH**, Keck AS, Wallig MA, West LG, Jeffery EH. Evaluation of the safety and bioactivity of purified and semi-purified glucoraphanin. *Food Chem Toxicol* 2008; **46**: 195-202 [PMID: 17804139 DOI: 10.1016/j.fct.2007.07.015]

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

Sincerely yours,

Signature: Kazutaka Yoshida **Date:** May 21, 2015

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