Author’s response to reviews

Title: Single and simultaneous effects of acrylamide and ethanol on bone microstructure of mice after one remodeling cycle

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Author’s response to reviews:

First of all, on behalf of my co-authors, I would like to express my deep gratitude for your interest in reviewing our article. We appreciate the time, energy and patience spent in reading our study, writing your comments and suggestions. We tried to accept all your comments and suggestions which have led to a significantly increasing quality of the manuscript. All changes made in our manuscript have been marked by yellow colour.

Reviewer 1

1. In relation to the Nomina Anatomica Veterinaria, anterior and posterior terms are used in human anatomy. Regarding to the animals, cranial instead anterior and caudal instead posterior terms are used in femur. Please, rewrite terms in the manuscript (page 5 - 114, page 7 - 149, 151, 155, 157).

This recommendation was accepted throughout the manuscript.
2. Is the voxel size written in relation to the uCT method (starting 5.2 mm and extending 1.5 mm; page 6 - 125, 126, 129, 130)? This information is interesting for readers.

We added following information in the manuscript: “High resolution scans with a voxel size of 6.8 um were acquired (70 kV, 200 μA, 300 ms, 0.5 mm, aluminum filter).” (Methods / Procedures section, page 6, lines 130-131)

3. Have you evaluated 2D analysis of trabecular bone?

No, we have not evaluated 2D analysis of trabecular bone.

4. I recommend to do also histological analyses of compact and trabecular bone which makes better view to morphological changes.

The primary aim of the 2D analysis is to investigate microstructural changes of bone tissue in a higher resolution and to analyse different parameters when compared to the micro CT. However, it’s almost impossible to do histological analysis of trabecular bone when bones are mineralized (they are not decalcified), unstained, according to the method used in our study. This method, however, allows to analyse 2D characteristics of compact bone and it is accepted worldwide. The 2D analysis of trabecular bone can be realized after decalcification of bones, embedding in paraffin (not in epoxy resin Biodur) and staining. On the other hand, decalcification-included methods often provide the same parameters as compared to the micro CT, e.g. trabecular number, trabecular width, trabecular spacing, trabecular area. Thank you very much for your recommendation, we are planning to do also histological analyses of trabecular bone in the future using different bone samples.

Reviewer 2

Abstract section:

Background: In the background section only the aim of the study was explained. It would be a good option to add some context to the study.

We added following information in the manuscript: “This study aimed to examine femoral bone microstructure of mice after single and simultaneous administration to acrylamide and ethanol since both substances are often consumed separately and/or together by humans.” (Abstract / Background section, page 2, lines 27-28)
Methods: The 4 groups of treatment should be named in the same way. For easy understand the text the control group may be cited as: "C group-control"

This recommendation was accepted.

We added following information in the manuscript: “Twenty clinically healthy adult mice were randomly divided into four groups following 2 weeks administration of toxins: A group - mice were fed with acrylamide (40 mg/kg bw); E group - mice were ethanol-fed (15 % ethanol); AE group - mice were simultaneously fed with both toxins, and a C group – control (without acrylamide and/or ethanol supplementation).” (Abstract / Methods section, page 2, lines 32-35)

I recommend to used the terms cortical bone (instead compact bone) and trabecular bone as they are the term used in most of the literature. If you consider my advise please check all the test.

This recommendation was accepted throughout the manuscript.

Methods section:

In my opinion it would be good for the reader that the division of the groups were explained again in methods section.

This recommendation was accepted.

We added following information in the manuscript: “Animals were randomly segregated into four groups following 2 weeks administration of toxins: A group - mice were fed with acrylamide (40 mg/kg bw); E group - mice were ethanol-fed (15 % ethanol); AE group - mice were simultaneously fed with both toxins, and C group – control (without acrylamide and/or ethanol supplementation).” (Methods / Animals section, pages 4-5, lines 93-97)

Page 5 line 96: In the control group must be explained if suffer some manipulation (as the administration of saline, vehicle…).

This recommendation was accepted.

We added following information in the manuscript: “Animals from C group received only physiological saline solution.” (Methods / Animals section, page 5, lines 101-102)
Authors must mention in the text which procedure used to administrate orally the toxics (free in water, probing…).

This recommendation was accepted.

We added following information in the manuscript: “Both toxins were dissolved in physiological saline and were administered orally to mice using a syringe in known doses.” (Methods / Animals section, page 5, lines 100-101)

Page 5 line 104: Author said that they put animals "under deep anesthesia" but later they related that they harvest the femora. It should be changed deep anesthesia for sacrifice.

This recommendation was accepted. (Methods / Procedures section, page 5, lines 107-108)

Page 5 line 104: Animals were sacrificed with a pentobarbital overdose. Did authors used any kind of sedation previous to death to avoid distress? If they used it please provide information. If they not please discuss why they don't se it (UE regulation (2010/63/UE) about the use of animals in research. In this regulation is described that the use of anesthetics for euthanasia must be accompanied by a previous sedation (loose of conscience).

We apologize for the inaccurate information in this section of the manuscript. We corrected it and added following information: “At the end of treatment period, mice were put into a state of deep anesthesia for sacrifice by Vetbutal (Biowet, Poland) administration in the amount of 35 mg/kg bw followed by rapid cervical dislocation and decapitation. Their femurs were next used for microstructural analyses.” (Methods / Procedures section, page 5, lines 107-110). The anesthetic was applied according to the manufacturer instructions. The procedure of mice euthanasia was approved by the First Local Ethical Commission on Experiments on Animals in Krakow (resolution no. 154/2014).

Page 5 line 110: Did author used some staining method for the 2D qualitative and quantitative assessment? If used, please report.

No, we didn’t use any staining.

Page 6 line 124: It is necessary to report the microCT parameters for scan and reconstruction.
We added following information in the manuscript: “High resolution scans with a voxel size of 6.8 um were acquired (70 kV, 200 μA, 300 ms, 0.5 mm, aluminum filter).” (Methods / Procedures section, page 6, lines 130-131)

Results:

In my opinion quantitative results of 3D analysis and from biochemical analyses should be represented in tables as well as in graphics. Please provide tables with numeric results.

This recommendation was accepted. We also provided tables with numeric results (Tables 2 -4).

Reviewer 3

In the following publications it is advisable to use lower concentration acrylamide and ethanol and repeat one (two) remodeling cycle in new publications.

Thank you very much for this recommendation. In the future, we are planning to use lower concentration of both toxins and repeat more remodeling cycles.

With great respect and appreciation,

Dr. Birgit Grosskopf