Author's response to reviews

Title: Bone turnover in passive smoking female rat: relationships to change in bone mineral density

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Author's response to reviews: see over
Re: MS: 2093265985500273 “Bone turnover in passive smoking female rat: relationships to change in bone mineral density” submitted to BMC Musculoskeletal Disorders

Dear Dr. Robert Layfield:

We are glad to resubmit you our revised manuscript after a minor revision according to referee #2 and referee #3. Meanwhile, a detailed response describing how we have responded to the specific points raised by the reviewers is also attached by the end of this letter. All changes made are highlighted in ‘green background’ of the revised text. For a more convenient review to you and the reviewers, I would like to upload our response in another file separately, as we upload the revised manuscript.

If you have any other concern regarding to our revised manuscript, please feel free to contact me. At the same time, we would like to appreciation you and the nice reviewers who had given us professional comments to improve our manuscript.

Best wishes.

Sincerely yours,

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Response to Reviewer #1 (Hermizi Hapidin): All of my previous questions and comments were answered properly by the author.

Answer: Thanks.

Response to Reviewer #2 (Cortino Sukotjo):

1. Page 3: please spell out the acronym of BMD and PCB on the page 3.
   Answer: Done accordingly (line 66, 77). BMD (bone mineral density) and PCB(Polychlorinated biphenyls).

2. Are you using: TRAP 5B or TRACP 5b? Please be consistent.
   Answer: Done accordingly (line 262, 266, 273, 278, 279, 523). ‘TRACP 5b’ has been used instead of ‘TRAP 5B’ or ‘TRAP 5b’

3. Page 5: how did you measure the nicotine concentration? Please add in the m and m
   Answer: We have added a more detailed introduction in the revised manuscript (line 126-133). The rats were sacrificed by intraperitoneal administration of sodium pentobarbital after 2, 3 or 4 months. Blood was collected from the abdominal vena cava into heparinized tubes and centrifuged at 1500 r.p.m. and 4°C for 5 min. Separated plasma was stored at -70°C for subsequent determination of blood nicotine concentrations. Nicotine concentrations in plasma were measured by gas chromatography with nitrogen-phosphorus detection. Sensitivity of the assay is 1 ng/ml nicotine.

4. Page 9: line 211: Hapidin et al..... (please explain more on the statement, for example: does the changes increased or decreased?)
   Answer: A detailed explanation of the reference has been provided in the revised manuscript according to your professional comment (line 215-218). Hapidin et al. demonstrated that 4 months of nicotine treatment was detrimental to bone by causing
an increase in the bone resorbing cytokines and cotinine levels and nicotine also exerted negative effects on the dynamic trabecular histomorphometric parameters.

5. Page 10: line 220: Ajiro et all .... (all the way to line 225, just before Cornuz).
The discussion on this ref was not significant.

Answer: The section has been deleted according to your comment (line 225).

6. Page 13, line 297-300: do you have any references on these statement?


**Passive smoking kills 6,800 annually**

Kyodo News

Roughly 6,800 people in Japan are estimated to die every year from lung cancer or heart disease caused by passive smoking, and more than half — around 3,600 — are exposed to secondhand smoke in their workplace, a research team said Tuesday.

About 4,600 of the victims are women, according to the team, which operates under the Health, Labor and Welfare Ministry.

"Huge harm is done," said Yumiko Mochizuki, a senior researcher at the National Cancer Center. "The government and employers need to recognize it is their responsibility to protect the health of working people."

Studies by the team and international organizations have shown passive smoking increases the risk of lung cancer and heart disease by 20 to 30 percent. The team's findings are based on this increased risk.

Based on a 2005 survey, the research team estimates that among 76 million nonsmoking adults — 48 million women and 28 million men — 30 percent of the women and 6 percent of the men are exposed to passive smoking in their homes, and 20 percent of the women and 30 percent of the men in their workplaces. The figures include those exposed to secondhand smoke both at home and in the workplace.

The team found in another study that passive smoking caused some 8 percent of
18,000 female deaths and 1 percent of 49,000 male deaths from lung cancer, as well as 9 percent of 34,000 female deaths and 4 percent of 42,000 male deaths from ischemic heart disease, which reduces blood supply to the heart.

The total number of victims in this study came to 4,600 women and 2,200 men, of whom 1,800 women and the same number of men were exposed to secondhand smoke in their workplaces, according to the team.

Response to Reviewer #3 (Malgorzata Brzoska):

The manuscript ‘Bone turnover in passive smoking female rat: relationships to change in bone mineral density’ by Gao S. et al. has been improved compared to its previous version; however some questions still need attention. The questions that should be addressed are listed below.

1) page 4, lines 82-83; markers of bone resorption are measured not only in the urine, but also in the serum
Answer: Done accordingly (line 83).

2) page 4, lines 83-86; this sentence needs revision.
Answer: The sentence has been revised as “Compston [19] showed that older smokers had high levels of bone resorption while early postmenopausal women had low levels of bone formation, though the mechanisms had not been clearly established.” (line 84-86).

3) page 6, line 129; it should be ‘…lumbar vertebrae (L4-L6) were…’
Answer: Done accordingly (line 133).

4) the results statistical analysis are still inappropriately interpreted. It cannot be stated that anyone parameter was decreased as a result of exposure to smoke when there was no statistically significant difference. For example, page 8, line 179 it can be read that smoke exposure resulted in a decrease in the BMD of the lumbar spine by 0.5%, 0.9%
and 11.8% after 2, 3, and 4 months, whereas in the next sentence we can read that the BMD at 2 and 3-month did not differ compared to the control. This same problem refers to the femur BMD and markers of bone turnover.

**Answer:** You are exactly right. The results section has been revised according to your professional comment (line182-201).

**Bone density characteristics**

BMD of the lumbar vertebrae and femur are demonstrated in Fig. 2. There was no significant difference between the lumbar spine BMD at 2-month or 3-month, but the lumbar spine BMD in the 4-month smoke-exposed rats was significantly lower than that in controls (P<0.01) (Figure 2). Similarly to the lumbar spine, these data on the femur BMD did not differ significantly at 2-month or 3-month, but the BMD of the femur was significantly lower in the smoke-exposed rats at 4 months (P<0.01) (Figure 2). Smoke exposure resulted in a decrease in the lumbar spine BMD by 11.8% and the femur BMD by 10.5% after 4 months, respectively, compared to control.

**Bone turnover**

Fig. 3 shows the results of the bone turnover measurements in the controls and smoke-exposed female rats. There was no significant difference in serum osteocalcin levels between smoke-exposed rats and controls (Figure 3). There was also no significant difference in the serum b-ALP activity and the TRACP 5b levels between 2-month smoke-exposed groups and the controls. However, significantly lower b-ALP activity and higher TRACP-5b levels were noted in the 3-month or 4-month smoke-exposed rats compared to controls (Figure 3). Smoke exposure resulted in a decrease in the serum b-ALP activity by 17.5% and 37.8% after 3 and 4 months, respectively, compared to control. Furthermore, passive smoking resulted in a increase in the TRACP 5b by 53.9% and 59.4% after 3 and 4 months, respectively, compared to control.

5) Page 9, lines 199-201; we can state that correlation (positive and negative) occurred only in the case when it is statistically significant.

**Answer:** The sentence has been revised as “Interestingly, b-ALP positively correlated
with BMD of the lumbar vertebrae ($r=0.764$, $P=0.027$, Figure 4) and femur ($r=0.899$, $P=0.002$, Figure 4) in 4-month smoke-exposed female rats.” (line 203-205).

6) Page 12, lines 261-262; what did you mean?

**Answer:** The sentence may not be appropriate and has been deleted (line 261).

7) Page 12, lines 281-285. In this site it should be discussed which components of the smoke may affect the skeleton.

**Answer:** The section has been revised (line 208-301). While many studies had focused on the hazardous effects of smoking on the skeleton, there was little direct evidence regarding which substance in cigarette exerted this effect. The major components of the smoke may affect the skeleton included carbon monoxide, nicotine and other toxic components of cigarettes (e.g., Cadmium, Lead, Chromium, Benzo(a)pyrene, dimethylbenz(a)anthracene). Loder et al.[41] reported that the incidence of congenital spinal deformations was directly related to carbon monoxide dose. In a previous study, nicotine given in the drinking water for 3 months was found to lower BMC in male mice [14]. Syversen et al.[42] showed that female Sprague Dawley rats were exposed to nicotine vapour for 2 years and there was no difference in BMD between control rats and nicotine-exposed rats. Berley et al.[43] and Yamano et al.[44] indicated that systemic nicotine may have a significant adverse impact on bone wound healing and inhibit the bone matrix-related gene expressions required for wound healing. Brzóska et al.[45] and Galazyn-Sidorczuk et al.[46] reported that chronic exposure to cadmium (Cd) or lead (Pb) through cigarette smoking had an injurious effect on the skeleton. Sankaramanivel et al.[47] revealed that a significant increase in the concentration of Chromium can decrease bone formation rate.

8) Page 13, lines 292-296; this text needs revision. The Authors should focus on what was revealed by Lee et al (not on what was done).

**Answer:** The text has been revised as “According to Lee et al. [42], BaP and DMBA can cause a loss of bone mass and bone strength, possibly through an increase in bone
turnover. Their results suggested that BaP and DMBA, two PAHs found in the tar fraction of cigarette smoke, may be in part responsible for the adverse effect of cigarette smoking on the bone[42].” (line 297-301).

9) Page 14, line 312; human may also be a passive smokers
Answer: The sentence has been revised as “Humans may be active smokers or passive smokers, and we cannot comment on the effects of active smoking using our model.” (line 319).

10) Page 14, lines 322-323; Is seems better to mention first that passive smoking increases bone turnover and next that it decreases bone mineral density.
Answer: The text has been revised as “increases bone turnover and then decreases bone mineral density” (line 329-331).

11) The English through the manuscript needs correction, there are numerous grammatical errors in the text, for example page 5, line 108-110: ‘…three control group and three smoke-exposed group. The smoke-exposed group were breed..’ .it should be ‘…three control groups and three smoke-exposed groups. The smoke-exposed groups were breed...’
Answer: The English through the manuscript has been revised (line 38, 59, 61, 62, 63, 64, 67,79, 85, 110, 111, 155, 160, 171, 173, 179, 306, 313, 324, etc).