Author’s response to reviews

Title: Atypical antipsychotics induce human osteoblasts apoptosis via Wnt/β-catenin signaling

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Author’s response to reviews:

Dear editors:

We are very excited to receive your letter and sincerely gratitude to the reviewer for valuable comments about our manuscript (PHAT-D-18-00073). Those comments are valuable and helpful for revising and improving our manuscript. We have studied comments carefully and have corrected them point by point, along with a clear indication of the location of the revision and marke it red. We would like to re-submit the manuscript to BMC pharmacyology & toxicology. We hope that this revised version is acceptable for publication in BMC pharmacyology & toxicology.

Note: Peifan Li and Yiming Wang are co-first authors, but Peifan Li ranks first in the author sequence, please refer to the author ordering section on the first page of Manuscript which is the
same as the previous version. (We find the Yiming Wang ranks first in the author sequence in your draft, page2)

Thanks again for your kind attention to this reply.

Looking forward to hearing from you.

Yours sincerely
Peifan Li

Point-by-b-point response

Manuscript ID: PHAT-D-18-00073

Title: Atypical antipsychotics induce human osteoblasts apoptosis via Wnt/β-catenin signaling

Authors: Peifan Li+, Yiming Wang*+, Xingde Liu*, Zhen Zhou, Jun Wang, Haiyan Zhou, Lei Zheng, Lixia Yang, Pingxia Xie, Da Zou

Reviewer #1:
Concern: Page 11 line 56 authors must explain how apoptosis rate was calculated; the valor on bar graph seems to be different than the value that appears on cell sorting graph? I got the same question for the drug tested.

Response: The apoptosis rate was automatically calculated by flow analyzer, and the mean value and variance are calculated based on the results of three independent experiments. The cell sorting graph is only the result of one flow analysis, while the bar graph is the statistics of three results. We have mentioned in the Statistical analysis section (page10, line17) that all the experiments were repeated at least three times. More explanations will be added in legend of figure 2 (page24, line14) and the method of apoptosis analysis (page7, line16).
Concern: Page 15 line 9 "However, the current study suggest that Aps may induce low BMD by sustaining elevate the secretion of prolactin and subsequently inhibit estrogen secretion as their dopamine D2 receptor-blocking effect" I don't see how the data presented in this article can demonstrate this effect?

Response: “Aps may induce low BMD by sustaining elevate the secretion of prolactin and subsequently inhibit estrogen secretion as their dopamine D2 receptor-blocking effect” is indeed not the result of our research, but the current view of most researchers in psychiatry on osteoporosis caused by APS, see reference [22].

Concern: Page 18 concerning the conclusion authors don't really demonstrated that b-catein variation observed with Aps treatment is responsive for the increases of apoptosis. The only things that authors can conclude with the presented data is that there is a correlation between the decrease of b-catenin in the nucleus and the increases of apoptosis but if authors want to demonstrated the hypothesis suggested in the cartoon more assays must be done to bring more evidence to confirm this hypothesis. For example authors claim that apoptosis is induced by the unbalance between bax/bcl2-MCl-1 that drive to the activation of caspase 3 but caspase 3 could be also activated by the extrinsic pathway via caspase 8, and it seems to me that this pathway must checked to improve the evidence of the involvement of bax/bcl-2 on the apoptosis activation. Analysis of caspase 9 (associated with the release of cytochrome C ) will be an another interesting evidence. For the more the use of resveratrol, a polyphenol, as wnt canonical pathway activator is not really relevant. Because Y. Zou in 2015 have published that resveratrol decreases the WNT canonical path way. More interestingly Chen HJ, in 2012 have demonstrated that resveratrol : "did not affect the accumulation and nuclear targeting of β-catenin. In contrast, co-immunoprecipitation and in vitro binding analyses substantiated that resveratrol was capable of disrupting the binding between β-catenin and TCF4, contributing to the decreased Wnt signaling." This important data tell us that nuclear β-catenin measure is not sufficient to conclude about the transcriptional activity of the Wnt canonical pathway. So to be sure that the anti-apoptotic effect of the resveratrol is due to the Wnt canonical pathway I suggest to measure apoptosis during the Aps/ resveratrol treatment in presence of wnt inhibitor, or test the transcriptional activity of β-catenin/TCF complex with transactivation assay (top flah). Without this data authors must modify their conclusions.

Response: Thank you very much for your valuable comments. This is a very interesting phenomenon. Three of the reports you mentioned confirm that resveratrol can reduce the activation of the Wnt/β-catenin pathway. We have also consulted a large number of related literatures and find that the literature about resveratrol-induced Wnt/β-catenin pathway inhibition is mostly concentrated in the field of tumor. However, in spinal cord injury (Wang HD, Br J Pharmacol, 2013), bone loss (Feng YL, Int J Mol Med, 2018 ) and Dental pulp stem cells (Feng G, In Vitro Cell Dev Biol Anim, 2016 ) In it, it was shown that resveratrol can promote the
recovery effect of WNT pathway in osteoblasts. Our study base on OB cells destruction, APs can cause OB cells apoptosis, which leads to Wnt/β-catenin pathway inhibition. Resveratrol can restore Wnt/β-catenin pathway activity through attenuating inflammation, depressing PPARγ signalling and activation of the Sirt1, and resist these adverse factors. However, our data does not directly prove that variation of β-catenin is responsive for OB cell apoptosis, We have modified our conclusions in the discussion (page17, line15) and legend of figure 5 (page25, line18).

Concern: Method for nuclear separation must be describe in materials an methods

Response: The Method for nuclear and cytoplasm separation have be added in Methods (page8, line5).

Concern: Page 8 line9 "were without treated….." this sentence is not clear.

Response: We have revised these sentences in Methods (page7, line9).

Concern: Page 8 line 12 'Risperidone (control) and treated with Risperidone at different concentrations…" authors must explicit exactly what they use as control in this form is nor really clear.

Response: We have revised these sentences in Methods (page7, line12).

Concern: Page8 line 59 why author speak about "ribo nuclease RNAse"?

Response: Ribonuclease (RNase) is a class of nucleases that catalyze the degradation of RNA into small fragments. The purpose is to reduce the interference of RNA in the experiment. However, we have modified it in Methods (page7, line19).

Concern: Page 10 line 28 " for Ku 70" what authors want to say?

Response: We have deleted this unclear description and corrected the method of Immunofluorescence analysis (page10, line2).

Concern: page 13 line 45 "similar result that " this sentence is not clear
Response: We have deleted this unclear description and corrected this sentence in Results (page13, line13).

Concern: Figure 2 B in the graph representation of cell sorting with respiridone the dose 5μM appear but in the bar graph is this value did not exist authors must correct the mistake.
Response: The corrected dose is 5μM, We have corrected it in Figure 2B and Results (page11, line1).

Concern: Figure 3 C magnifications must be specified. In material and method the type (optical, apotome, confocal) and the reference of the microscope must be specified because some device don't allow to measure a real colocalisation.
Response: We have corrected it in Figure 3C and rewritten the immunofluorescence method according to your request in Methods (page9, line1).

Concern: What is the Aps concentration used in human therapy (or serum concentration detected) and what's the concentration used in the culture cell during this assay? is this value is in the same range?
Response:
As Aps is a type of relatively safe antipsychotics, the serum concentration of Aps in patients are rarely tested at the conventional therapeutic dose. The safe serum concentrations of the four Aps were not determined, and the results obtained in the experiment were inconsistent, roughly between 0.3 and 1.2 μM. The change of protein caused by Aps at the concentration within the safe range may be very small and cannot be detected. In order to make a significant difference in our experiment, we chose the concentration of OB cells IC50 (Olanzapine (40 μM), Risperidone (40 μM), Amisulpride (30 μM) and Aripiprazole (10 μM)). In future in vivo experiments in animals, we will consider the safe blood drug concentration range more.

Reviewer #2
Concern: In the abstract, this sentence is not clear to me: "We cultured human osteoblast cell line hFob1. 19 (OB) treatments with olanzapine, risperidone, amisulpride, aripiprazole or resveratrol combined with one of APs in vitro." Without further explanation I would understand it in a way
that these substances are the APs. But why then combine them with the APs? And what has resveratrol to do in this list?

Response: This sentence is just to show which drugs are used in the experiment. We have revised this sentence in Abstract (page2, line7).

Concern: English language editing is needed.
Response: We have asked professors whose native language is English to examine language.

Concern: It should be better and earlier motivated/explained why resveratrol was chosen.
Response: We have added this content why resveratrol was chosen in Background. (page5, line11)

Concern: In the manuscript its nowhere explained what the CCK-8 assay actually is.
Response: The full name of CCK8 is Cell Counting Kit-8, and its full name and laboratory procedures had added in Methods (page6, line11 and page7, line7).

Concern: The FACS assay for determining apoptosis needs to be explained better.
Response: We have modified the method of apoptosis (page7, line17).

Concern: Results: "We found that β-catenin protein expression increased compared with control group (Fig.3 A)." I think the authors what to say that it decreases.
Response: We have corrected it (page13, line10).

Concern: It would be helpful to have an immunofluorescence imaging based analysis of the different subcellular localization of β-catenin depending on the presence of the APs and resveratrol.
Response: I am very grateful to the reviewers for their suggestions. Based on your suggestions, we have revised the results (page13, line11), but the analysis column of fluorescent grayscale is not added in the Figure.