Author's response to reviews

Title: Bone Growth during Rapamycin Therapy in Young Rats

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Editor
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Dear Editor:

Enclosed with this letter is the revised version of our original article entitled “Rapamycin reduces bone growth, decreases angiogenesis and lowers chondroclastic activity in young rats” for consideration for possible online publication in the BMC Pediatrics Journal. The title has been changed and the reviewers’ queries and suggestions have been answered. We would like the reviewers for their careful critiques and important suggestions.

If accepted, we would like to request that Pubmed has an access to the accepted online version.

Thank you for attention to this matter. I hope you will consider our paper for publication.

Sincerely,

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Answers to Reviewer’s Comments:

Reviewer #1

1. Page 8, paragraph 2. Regarding the increase in creatinine levels observed, perhaps this point should be clarified by stating that the rise in creatinine was observed in both controls and RAPA-treated animals, at 4 weeks.

A statement regarding the serum creatinine levels was added to the revised paper.

2. Page 8, paragraph 3, fig. 1. It will be difficult for the inexperienced reader to estimate hypertrophic zone (HZ) heights, looking at the images presented in Fig. 1A and 1B. Thus, it should be indicated in the text exactly how the top and the bottom of the hypertrophic zone were identified, in order to measure its height. It also might be possible to superimpose dashed lines at the top and bottom of the HZ in the images of growth plates presented here, to make the point that the HZ was higher with RAPA treatment.

Brackets have been added to the revised paper.

3. The RAPA-treated HZ shown in fig. 1B doesn't look significantly higher than control. Was the difference in HZ height at 4 weeks not significant in control vs RAPA-treated growth plates? Are the magnifications of photomicrographs in Fig. 1A the same; (because the control growth plate looks slightly more magnified)?

There was no difference in the measurements of the growth plate, proliferative zone and hypertrophic zone between the Rapamycin and Control groups at 4 weeks. The magnification of the growth plate cartilage in both 2 weeks and 4 weeks are the same.

4. Page 11, line 3, Fig. 4B. Although the text states that "IGFBP3 protein expression was localized to the proliferating chondrocytes", examination of Fig. 4B suggests that there was much IGFBP3 expression in hypertrophic chondrocytes of both RAPA-treated and control growth plates, at both 2 weeks and 4 weeks.

We agree with the reviewer that the IGFBP3 expression extended into the upper hypertrophic chondrocytes in both Rapamycin and Control groups. This statement was added to the revised version of the paper.

Reviewer #2

1. Immunohistochemistry and in situ hybridization are good techniques to assess the pattern of distribution of a given protein and mRNA, respectively. However, these techniques are not strictly suitable for quantification and have a limited value to assess small – moderate differences in the degree of expression.

We agree with the reviewer that ISH and IHC provide qualitative rather than purely quantitative evaluation however, we have shown that the differences
between the rapamycin and control groups were quite significant in the current experiments. We also agree that succeeding studies should include either laser microdissection in order to separate the layers of the growth plate cartilage or DNA microarrays.

2. The authors should clarify why food efficiency ratio of animals treated with rapamycin was higher than that of control rats if the groups were pair-fed and control rats gained more weight after 2 and 4 weeks.

There was an error in the previous values. The correct values have now been included in the revised table (Table I) version.

3. The inclusion of untreated control group fed ad libitum would be useful for the understanding of changes seen at the growth plates.

We agree with the reviewer that an inclusion of a group fed ad-libitum would have been useful in order to evaluate the effect of caloric restriction but we thought that a pair-fed control group would yield more information in this experiment.

4. The amount of chow eaten by the different groups of rats should be provided, just to assess the degree of reduction in food intake.

We did not include the total gram of food intake per group since majority of nutrition studies use either food efficiency or caloric efficiency (grams of weight gain/grams of food intake) to compare the effect of nutrition on body growth.

Reviewer # 3

1. The authors use rats given oral rapamycin for 2 or 4 weeks and evaluate numerous morphometric parameters and the expression of an array of proteins. While many of the results are intriguing, the authors extend their conclusions beyond the scope of their data. For example, the title states that rapamycin lowers chondroclastic activity yet the data shows no difference in OPG expression, RANKL is transiently depressed at 2 weeks but is restored at 4 weeks, and a decrease in the number of TRAP stained cells (this last is supportive of the statement). They do not actually measure anything that directly measures resorption activity and the OPG and RANKL data do not support the claim. This is just one example and there are several others throughout the manuscript [such as defining that the rapamycin treated animals need more calories to grow which can only be stated if in fact additional calories were provided and the animals grew--- unlikely since those animals had free choice access to food; this is stated in the results too]. The authors are encouraged to stay within the scope of their findings.

We have revised the discussion to reflect changes as suggested by the reviewer.

2. Though rapamycin has a clear effect on many of the parameters after 2 weeks of treatment (from 3-5 weeks of age), the rapamycin effect is gone for many of the measurements by 4 weeks of treatment. Though the study concluded after 4
weeks, one is left wondering whether the animals would exhibit catch up growth. That should be, at the very least, considered in the discussion that the effects of rapamycin may only be transient. If the growth retardation remained throughout development, that gives additional impact to the authors’ study. The authors do not give proper thought to the 4 week findings. The absence of data from 4 weeks in the abstract emphasizes this concern. The authors also equate provision of rapamycin for 4 weeks vs 2 weeks (starting at 3 weeks) to providing rapamycin at a later developmental age in the abstract and discussion. This is not accurate.

The abstract and the discussion have been changed to include the reviewer’s comments.

3. Both the tibia and femur were collected but it would appear (though the figure legends do not state that but the methods do) only the tibiae were used for the IHC and in situ hybridization. Why?

The Materials section has been revised since we only used the tibia for the current study.

4. The images depicted in figure 3 do not support the data analysis presented.

This has been corrected.

5. Figure 4 does not support the statements the authors make in the results: IGFBP3 does not decrease 44% after 4 weeks of rapamycin as stated. The data presented in Figure 4 of a smaller hypertrophic zone does not fit with the data in Figure 1 that reports 2 weeks of rapamycin increases the hypertrophic zone.

The statement has been changed. IGFBP3 expression decreased in the 2 weeks rapamycin group compared to the 2 weeks Control animals and 4 weeks Rapamycin group.

6. The lack of concordance between the rapamycin dose used and what is given to children is a concern especially since many of the measurements were back to control levels by 4 weeks of treatment. Is this really a good model for a human study?

Although we used a higher dose of rapamycin in the current study compared to the pediatric dose used in renal transplant recipients, the popularity of rapamycin as an immunosuppressant in transplant and other immunologic diseases has been increasing. Currently, we do not have enough information whether growth will be affected if rapamycin is given to patients at a very early age (neonates). Subsequent studies need to be done to answer these questions.

7. The discussion lacks substance.

Parts of the Discussion have been changed to include the reviewer’s comments.

8. In Abstract/Methods section: “anthropometric” refers to the measurement of humans. The authors are measuring rats.
This has been changed to anthropomorphic.

9. In Abstract/Methods section, last sentence the word “for” should be omitted: …“to evaluate for …”

This has been changed.

10. In Background: should be BrdU

This has been changed.

11. In Methods: should include whether both left and right bones were harvested, were both measured and average length used or?? What were the anatomical landmarks for measurement? What is the replicate number per animal or was a single section used per animal? Were all measurements and assays done using the proximal tibial growth plate?

The methods section has been changed to include the reviewer’s comments.

12. In Methods: define what is meant by "50-60 cell profiles" were assessed. Is that 50 or 60 cells of a given region or ? Also define the area used for the reporting of TRAP positive cells: total area or surface area at the chondro-osseous junction (the latter being more relevant).

This is clarified in the revised version of the paper.

13. In Methods: why was a one way ANOVA used rather than a multifactorial analysis used?

We have revised the statistical discussion to include the t-tests and ANOVA when appropriate.

14. In Results: the authors note a positive correlation between PTH and IGF-1 is serum (not expression as the authors state) which is difficult to reconcile given that at 4 weeks of age rapamycin decreases PTH and increases IGF-1. But the authors are only focusing on the 2 week treatment period and neglecting the 4 week.

The correlation was significant between iPTH and IGF-1 serum levels for the 2 and 4 weeks Control group. This statement was deleted in the revised paper.

15. In the Tables need to state that the data are presented as means + 1 SD. The “anthropometric” in Table 1 title needs to be changed. The statistical significance designation is unclear.

The changes have been indicated in the Tables.

16. The figures are poorly presented. As in the tables, the statistical significance designation is unclear. The figures do not have the a, b, and c panel designations alluded to in the text. The presentation between table and figures differs with the ordering of the treatments (e.g., main category weeks in tables but rapamycin vs
control in figures).

The figures have been amended to include the letters. The statistical significance has been added to the Legend Text. We have decided to change the order of presentation for the Figures (and not the Tables) in order for the reader to be able to compare the pictures from the same groups side by side.

17. The results section should contain only results; there are elements of discussion within the results.

The Results section has been revised.

Reviewer # 4

1. The authors use Weanling rats (3wks of age, treated to age 5 weeks and 7 weeks): what is the human equivalent to that? Are the 5 week old rats adolescents and the 7 week old rats adults? So that we can extrapolate to where the growth data might apply?

These are the approximate human equivalents: 3 weeks or 10-21 days old rats (infant), 5 weeks or 21-45 days (younger child), 7 weeks or 21-45 days (older child). A statement has been included in the Methods Section of the revised paper.

2. The authors talk about the "hypertrophic zone" in the growth plate cartilage. They should explain what that area means/signifies at the first mention of the term: as it pertains to the whole paper (discussion/results etc).

A statement has been included regarding the importance of the hypertrophic zone in endochondral bone growth in the Discussion part of the revised paper.

3. They looked at the tibial growth plates, is that the best place to look for growth? If so, they might want to mention the preceding work done to demonstrate that.

Using oxytetracycline labeling to evaluate bone growth and bromodeoxyuridine incorporation to evaluate chondrocyte proliferation, Wilsman and colleagues have demonstrated that the proximal growth plate in the tibia, produced 4 times as many chondrocytes per day compared to other growth plates [1]. We have always used the proximal growth plate in majority of our experiments because of faster bone growth. A statement has been included in the Methods section of the revised paper.

4. In Methods: is the term "cell profile" (page 5) clear, does it need to be defined?

The term has been explained in the revised paper.

5. The Results section needs the most clarification: Throughout the explanation of the results, the authors mention percents of change while referring to the tables. The tables however have raw data, no percents are noted. For example, tables 1 and 2 are clear, but perhaps they need another column or table to show the percents referred to in the body of the text.
We thought that showing the raw data for the Tables and Figures will give more information to the reader. We did not include the percentage since the data will be redundant if already included in the text.

6. Clarify Food Efficiency Ratio

Food Efficiency or caloric efficiency has been used in various nutrition studies including renal failure to evaluate the relationship between caloric intake and weight gain [2, 3]. This has been included in Table I including the units used.

7. For the photomicrographs, perhaps it would be good to clearly delineate in Figure 1, where the normal proliferation zone is, where the hypertrophic zone is, to orient the reader.

Brackets have been added to Figure 1.

8. The In-Situ Hybridization and Immunohistochemistry Studies section: Needs editing as there is a lot of introduction/discussion type material that may best have been introduced earlier (i.e at the end of page 6). This is a results section, and the results get lost in all the wording.

The results section has been revised and the introductory wording has been deleted.

9. In the text, when talking about Ihh expression (page 10), they say "Ihh expression confined mostly to the hypertrophic chondrocytes increased approximately 2-fold after 2 weeks of rapamycin (Figure 3a)" but in the legend of that Figure, it seems to jive more with Figure 3B.

This has been corrected in the text.

