Author’s response to reviews

Title: Genistein Treatment Improves Fracture Resistance in Obese Diabetic Mice

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RESPONSE TO REVIEWERS

AUTHORS: We thank the reviewers for their suggested improvements to the manuscript. We appreciate the effort that went into these careful and thoughtful reviews of our manuscript. In the following, we have attempted to address the concerns of the reviewers. The changed parts in the revised manuscript have been marked with red font. We sincerely believe that all the changes suggested by the reviewers helped us improve the manuscript and we are tremendously grateful.

REVIEWER 1

Reviewer reports:

The reviewers found this work of significant value, please see the comments below and respond with changes in the text or explain as needed. If a larger sample size is available, please provide further analyses.

Reviewer #1: Summary: Odle et al explored whether the increased risk of fracture in type 2 diabetics would benefit from genistein supplementation by treating Ob/Ob and control mice for 4 weeks and assessing the bone by histomorphometry and 3 point bending. The authors found that although there was an increase in B.Ar and T.Ar in Ob/Ob mice, the B.Ar/T.Ar was the same when compared to control mice. They found that Genistein decreased Tt.Ar and increased ultimate force in both the Ob/Ob and control mice and attribute these alterations to changes in bone quality. Although it is important to assess potential treatments in type 2 diabetics, I have concerns over statistical power and in the types of assessments utilized to assess the bone parameters.
Are the methods appropriate and well described?

* It may be better to perform a volumetric assessment of bone using a method such as uCT to assess both cortical and trabecular bone parameters in all treatment groups. By assessing volumetric parameters of trabecular bone and cortical bone, you may better observe differences in bone parameters.

AUTHORS: We agree such data would provide insight and we are seeking funding for uCT but currently do not have the resources to collect these data. Instead, we added to the Discussion section of the manuscript:

Further study is required to determine the exact effects of genistein on bone geometry and composition to fully explore this hypothesis. Such studies should include micro-CT data, given phytoestrogen treatment has been shown to prevent loss of three-dimensional bone microarchitecture [50, 51]. Volumetric data may further explain the improved resistance to bending demonstrated in genistein-treated samples.

Are the conclusions drawn adequately supported by the data shown?

* Histomorphometric assessments typically require a large sample number (10-12) in order to have adequate statistical power. I am concerned that the analysis is not powered appropriately to observe a difference. This is important given that you suggest the treatment may affect overall bone structure since you don't observe differences in the histomorphometry. Please do a power analysis to show that your study has sufficient power to detect a difference. Otherwise, please increase the sample numbers to boost your power.

AUTHORS: We conducted power and effect analyses using the general linear model procedure in SPSS prior to submission. We also conducted tests to ensure we do not violate the assumptions of the statistics we use, in this case the Kolmogorov–Smirnov test for normality and homogeneity tests appropriate to our ANOVA. The results of these tests indicate we have robust, parametric data and the test of power indicates our negative findings are not likely to be type II errors. We added to the Methods:

Tests of power, normality and homogeneity of variance show our analyses have adequate power to avoid type II errors and do not violate assumptions of the statistical analyses.

Other comments:

* Is treatment with Genistein for 4 weeks enough time to observe differences in the bone?

AUTHORS: Our lab, and others, have found significant differences among treatment groups using a 4-week treatment period. We modified the Methods section of the manuscript describing the treatment to highlight this point with citations:
This diet is commonly used to study the effects of phytoestrogens on the T2DM condition in mouse models is comparable to human soy-based diets and has been shown to have significant physiological effects with four weeks of treatment [22, 25-28].

* As genistein has been shown to have effects on both the osteoblast and osteoclast, a histomorphometric assessment of bone turnover (toluidine blue and TRAP stained sections) and analysis of bone mineralization (von kossa staining) would add to the bone analysis

AUTHORS: The inhibitory effects of phytoestrogen treatment on resorption have been well documented with TRAP staining and other methods in a manner that support our findings. We added to the Discussion section:

Additionally, approaches that highlight the osteoclast-inhibiting effects of phytoestrogens should be used to assess how phytoestrogenic suppressive effects on bone resorption correlate with fracture resistance. These effects have been identified in other models, but not in the T2DM model [52, 53].

* If you think the treatment alters bone quality, you could look at the intrinsic biomechanical properties from the 3-point bending analysis (cross-sectional moment of inertia and elastic modulus for example) to look at intrinsic material properties.

AUTHORS: This is an excellent suggestion. We have previously published cross-sectional data using this model for T2DM (Michelin et al., 2016) that are cited in the manuscript. We agree mechanical property data would be beneficial because they are lacking in the literature, however our current setup does not provide elastic modulus or Poisson’s ratios. We could estimate these properties from a load-displacement curve, but due to their non-linearity in the elastic range they would not have suitable accuracy for publication. To compensate, we added a figure displaying the load-displacement relationship by treatment group. The following was added to the Figure Legend:

Fig. 4. Load-displacement curves derived from three-point bending test results. The test was performed on femora of lean mice fed a standard diet (Lean STD), lean mice fed 600 mg genistein /kg diet (Lean + GEN), obese mice fed a standard diet (Obese STD), and obese mice fed 600 mg genistein /kg diet (Obese + GEN).

Reviewer #2: The impact of obesity and type 2 diabetes on the skeleton is a significant concern, and the paper investigates an interesting and relevant topic. The animal model is also interesting, and genistein as a phytoestrogen is an appropriate dietary supplement to investigate. The following minor issues should be addressed:

1. The abstract should include the duration of treatment (4 wks) and the number of animals per group (n=5)

AUTHORS: We added the requested information to the abstract.
2. Genistein treatment appears to shorten the femora of db mice. Presumably this is due to some effects on the growth plate. Could the authors provide some histological images of growth plates in treated vs control mice, or in vitro data on proliferation and/or differentiation of genistein-treated chondrocytes?

AUTHORS: We cite our previous papers (Cooley et al., 2015, Michelin et al., 2016), among others, that show histological images of the growth plate highlighting the effects of phytoestrogen treatment. We modified the Discussion to read:

While the precise mechanism that inhibits longitudinal limb bone growth is unknown, genistein and other phytoestrogens have been demonstrated to effect the thickness, calcification, and chondrocyte proliferation of limb bone growth plates, as shown in previous studies [22, 23, 32, 33].

3. Representative force-displacement curves should be added, and also data on post-yield mechanical properties.

AUTHORS: Unfortunately, our current setup does not provide post-yield mechanical properties. We did add a figure to the manuscript showing load-displacement curves for each treatment group. We could estimate mechanical properties from the curves, but due to their non-linearity in the elastic range they would not have suitable accuracy for publication. The following was added to the Figure Legend:

Fig. 4. Load-displacement curves derived from three-point bending test results. The test was performed on femora of lean mice fed a standard diet (Lean STD), lean mice fed 600 mg genistein /kg diet (Lean + GEN), obese mice fed a standard diet (Obese STD), and obese mice fed 600 mg genistein /kg diet (Obese + GEN).