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BLUEBERRY POLYPHENOLS DO NOT IMPROVE BONE MINERAL DENSITY OR MECHANICAL PROPERTIES IN OVARIECTOMIZED RATS

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Abstract

Introduction: Osteoporosis-related bone fragility fractures are a major public health concern. Given the potential for adverse side-effects of pharmacological treatment, many have sought alternative treatments, including dietary changes. Based on recent evidence that polyphenol-rich foods, like blueberries, increase calcium absorption and bone mineral density (BMD), we hypothesized that blueberry polyphenols would improve bone biomechanical properties.

Methods: To test this, 5-month old, ovariectomized Sprague-Dawley rats (n=10/gp) were orally gavaged for 90 days with either a purified extract of blueberry polyphenols (0-1000 mg total polyphenols/kg bw/d) or lyophilized blueberries (50 mg total polyphenols/kg bw/d). Upon completion of the dosing regimen, right femur, right tibia, and L1-L4 vertebrae were harvested and assessed for bone mineral density (BMD), with femurs being further analyzed for biomechanical properties via three-point bending.

Results: There were no differences in BMD at any of the sites analyzed. For bone mechanical properties, the only statistically significant difference was the high dose group having greater ultimate stress than the medium dose, though in the absence of differences in other measures of

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AUTHOR CONTRIBUTIONS

DPC and CMW designed the primary study, and KMGH contributed to the design of this ancillary analysis. CMW was PI for grant funding the research; DPC performed all animal procedures and BMD analysis; EAS and MRA performed the bone mechanical tests and analyzed these data. DPC, EAS, and KMHG drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

Dennis Cladis, Elizabeth Swallow, Matthew Allen, Kathleen Hill Gallant, and Connie Weaver declare that they have no conflicts of interest.

ETHICS APPROVAL

Animal experiments were conducted in adherence to Purdue University Animal Care and Use Committee (PACUC) guidelines, following an approved protocol (1808001790).

bone mechanical properties, we concluded that this result, while statistically significant, had little biological significance.

Conclusion: Our results indicate that blueberry polyphenols had little impact on BMD or bone mechanical properties in an animal model of estrogen deficiency-induced bone loss.

MINI ABSTRACT

Alternatives to osteoporosis medications are increasingly sought by patients. Recent evidence suggests polyphenol-rich foods, like blueberries, improve bone health. Here, we report that blueberry polyphenols did not alter bone breaking strength in estrogen-depleted rats, indicating that they may not reduce osteoporosis-related fractures.

Keywords

Blueberries; polyphenols; bone mechanical properties; osteoporosis; BMD

INTRODUCTION

Age-related bone loss weakens bone and leads to osteoporosis in many individuals. Treatments for osteoporosis aim to decrease fracture risk, which involves not only increasing bone mass but also bone mechanical properties.[1] Pharmacological treatments, including antiresorptive and anabolic agents, are commonly prescribed to individuals with osteoporosis to slow bone loss or build new bone mass.[2] However, due to adverse side effects and the desire for non-pharmacological treatment options, many have sought alternative treatment options, including dietary interventions.[1] Historically, most attention has been given to diets rich in essential nutrients (e.g., calcium and vitamin D), though in recent years, bioactive ingredients (e.g., fiber and polyphenols) have also shown beneficial impacts in limiting age-related bone loss.[3]

In recent years, interest in using polyphenol-rich foods, like blueberries, to alleviate bone loss has increased. Mechanistic studies have shown that polyphenols can mitigate the rapid bone resorption that occurs in postmenopausal females by stimulating osteoblastogenesis and inhibiting osteoclastogenesis.[4] By targeting these mechanisms, the rate of bone mineral density (BMD) loss has been decelerated in both clinical and preclinical models.[5] Because of this, people are incorporating polyphenols into their diets and using dietary supplements to consume larger doses of polyphenols.[6] In fact, 1 in 3 US adults use polyphenol-rich botanical supplements, with females 51-70y as the highest consumers.[7, 8] This is the life stage when many females go through menopause and postmenopausal bone loss. Therefore, understanding the potential for polyphenols to mitigate postmenopausal bone loss and, ultimately, fracture risk, has clinical significance.

To address this, our lab has undertaken a series of human and animal studies, showing that blueberry polyphenols positively impact calcium absorption and BMD in postmenopausal females as well as ovariectomized (OVX) rats and mice.[9-13] Our results, as well as those from other studies, indicate that polyphenols improve several skeletal properties. However, there are limited data on mechanical properties, which are pre-clinical measures more closely correlated with clinical fracture than surrogate measures like BMD.[14]

Although surrogate measures of bone health are important, fragility fractures are the key clinical outcome of importance. Therefore, to assess the impact of blueberry polyphenols on bone mechanical properties, we analyzed bones from OVX rats that underwent a 90d blueberry toxicity study in which we demonstrated that blueberry polyphenols are safe up to 1000 mg total polyphenols/kg/d,[15] though there were clear differences in blueberry phenolic metabolism between groups.[16] We hypothesized that blueberry polyphenols would improve bone mechanical properties following a 90d treatment.

MATERIALS AND METHODS

Animal experiment

The full details of the animal experiment, including rationale for dosing regimen and doses selected, are presented elsewhere.[15, 16] Here, we present a brief overview of the animal experiment, treatment regimen, and methodological details on bone analyses and then detail the bone analyses that were specifically carried out for this ancillary study.

Five-month old, virgin female Sprague-Dawley rats underwent OVX and were allowed to recover for one month before beginning treatment. The OVX model was chosen as an established model of postmenopausal bone loss.[14] All animals were maintained on a polyphenol-free diet (soybean oil replaced with corn oil) throughout the recovery and study periods to eliminate confounding from soya-derived isoflavones, which act as phytoestrogens.[17] Animals then underwent 90d of dosing via oral gavage in one of 5 treatment groups (n=10/gp). Rats received an oral gavage of either purified blueberry polyphenols containing 0, 50, 250, or 1000 mg total polyphenols/kg bw/d (designated water, low, medium, and high, respectively) or lyophilized, whole blueberries containing 50 mg total polyphenols/kg bw/d (designated BB). Gavage doses were prepared as slurries of distilled water mixed with purified blueberry polyphenols (VitaBlue Pure American Blueberry Extract, FutureCeuticals, Momence, IL, USA) or lyophilized composite wild blueberries (*Vaccinium angustifolium*, Wild Blueberry Association of North America, Old Towne, ME, USA). Purified blueberry polyphenols were prepared from an aqueous extract of whole blueberries and spray-dried with a maltodextrin carrier, while the lyophilized composite blueberries were freeze dried and milled into a powder. The total phenolic content was 26.7% (w/w) and 3.52% (w/w), respectively. Details on the complete phenolic characterization was published previously.[15] Upon completion of the 90d dosing regimen, animals were euthanized via CO_2 asphyxiation and the right femur, right tibia, and L1-L4 vertebrae harvested from all animals. Bones were scanned for BMD (see below) before being wrapped in saline soaked gauze and frozen at −20°C until analysis of bone mechanical properties.

Bone Analyses

Harvested femur, tibia, and L1-L4 vertebrae with soft tissue removed were analyzed for bone area, bone mineral content (BMC), and bone mineral density (BMD) using a PIXImus 2 mouse densitometer (GE Lunar PIXImus).

Mid-diaphysis femur microarchitecture was assessed via micro-CT (Skyscan 1176, Bruker, Madison, WI) with a 9μm voxel size and a 0.5 aluminum filter. Scans were reconstructed, rotated, and analyzed with Bruker Software. Cortical parameters were measured in mid-shaft femur slice calculated by half of femur length that was measured with calipers. Standard measurements and nomenclature were used to report results. [18]

To assess whether blueberry polyphenols had any effect on the structural or material mechanical properties of bone, whole femurs were subjected to three-point bending with monotonic loading to failure using standard methods.[19, 20] Prior to mechanical testing, femora were thawed to room temperature. Supports were set 9mm apart (18mm bottom span) and femora were positioned with the posterior surface in tension. All bones were kept hydrated with saline and pre-loaded to approximately 0.5N. Bones were loaded at a 2mm/min displacement rate (TestResources, Shakopee, MN) and data collected at 10Hz. Standard bending definitions were used to determine structural mechanical properties from load-deformation curves and material properties were calculated using standard equations in a MATLAB script, as previously described.[20, 21] Mechanical data are presented using standard nomenclature.[19]

Statistics

Statistics were completed using SAS (SAS Institute, Raleigh, NC). Differences between dose groups were analyzed via one-way ANOVA. When data were not normal, they were transformed using appropriate transformations prior to statistical analysis. Post hoc analyses were carried out using Tukey's HSD test, with significance defined as $\alpha = 0.05$.

RESULTS

At sacrifice, there were no significant differences between treatment groups for bone area, BMC, or BMD at any of the three sites analyzed by PIXImus (Table 1). Further examination of cortical architecture, at the mid-diaphysis of femurs by micro-CT, showed no significant differences in bone area or cortical thickness (Table 1).

There were no differences in bone structure-level mechanical properties in rat femurs, including ultimate force, total displacement, stiffness, or total work (Figure 1a). Aside from a small but statistically significant difference in ultimate stress between the high and medium dose groups there were no other differences in ultimate stress, total strain, modulus, or toughness (Figure 1b) across the groups.

DISCUSSION

Our results showed that blueberry polyphenols did not improve BMD, microarchitecture, or mechanical properties of bone in OVX rats stabilized following ovariectomy when administered daily for 90d. These results were consistent for both purified blueberry polyphenols (as may be present in dietary supplements) as well as lyophilized whole blueberries (mimicking dietary consumption). The BMD values at the three sites analyzed were consistent with other studies.[14] Based on the null results observed in bone properties measured in this study as well as the absence of biological or physiological differences as

previously reported,[15] we concluded it was unlikely differences in mechanical properties would be observed at other sites.

Previous studies using blueberries demonstrated that they may have benefits to bone health in OVX rats and mice as well as post-menopausal women.[9-13] These studies demonstrated that moderate doses of blueberry polyphenols increased calcium retention, which may lead to improvements in bone strength. In an acute study, a single, high dose of blueberry polyphenols (1000 mg polyphenols/kg) increased calcium absorption in OVX rats.[9] A similar study showed that moderate doses of blueberry polyphenols (5% (w/w) lyophilized whole blueberries in diet) increased calcium retention in OVX rats over 10d.[10] A follow up study feeding the same diet to OVX rats for 8 weeks showed that blueberries increased calcium absorption and bone deposition, with a trend ($p=0.08$) towards mitigating trabecular bone loss in comparison to control diet animals.[11] Interestingly, a longer study of 100d demonstrated that lyophilized blueberries (5% (w/w) in diet) improved BMD in OVX rats.[22] In a clinical study of postmenopausal women, moderate doses of lyophilized blueberry powder (17.5-35 g/d) increased bone calcium retention. However, when increasing the dose to 75 g/d, bone calcium retention was no different than during the control phase without blueberries.[12] Finally, in a study of OVX mice, lyophilized blueberries (10% (w/w) in diet) prevented OVX-induced deficits in BMD.[13]

Each of these previous studies showed improvements in surrogate measurements of bone strength, but they did not evaluate the impact of blueberry phenolics on mechanical properties or fracture. This is an important distinction, as most surrogate measures of bone strength (e.g., BMD and calcium absorption) are only partially correlated with bone mechanical properties and fracture. BMD, for example, only explains about 60-70% of bone strength and is not always an accurate predictor of risk of fragility fractures.[14] Thus, the current investigation builds upon these surrogate measures by assessing bone strength and resistance to fracture via micro-CT and three-point bending, which has direct clinical relevance to osteoporotic fractures.

In the few studies that have evaluated mechanical properties, results are mixed. For example, one study feeding lyophilized grape powder to OVX rats (25% diet (w/w)) for 8 weeks showed no change in BMD but did show a significant increase in cortical thickness and ultimate breaking force. This indicated that grape polyphenols did not influence surrogate measures of bone quality (e.g., BMD or calcium retention), but did improve bone strength and resistance to fracture.[23] Another study incorporated olive oil (50 g/kg diet) or oleuropein (the main bioactive polyphenol in olive oil; 0.15 g/kg diet) in the diet of OVX rats for 80d. There were no differences in BMD or mechanical properties in rat femurs after completing treatment, though the authors noted that when inflammation was chemically induced in a separate group of OVX rats 3 weeks prior to sacrifice, both olive oil and oleuropein mitigated inflammation-induced bone losses.[24] A study adding dried plums to the diets of OVX mice for four weeks found that doses of 15% and 25% (w/w) significantly increased vertebral BMD and BMC. This was followed up by a computer-simulated vertebral compression test, which predicted that these same doses increased mechanical properties, including strength and stiffness.[25] Finally, a study incorporating yerba mate into the water of OVX rats (25 g/L, brewed at 90°C, then filtered prior to

administration), found no differences in tibia BMD or bone mechanical properties (assessed via 3-point bending) between water and yerba mate groups, leading to the conclusion that polyphenols in yerba mate had little effect on bone strength.[26] Taken together, these studies demonstrate the heterogeneity of polyphenol sources and their subsequent impact on bone mechanical properties.

Our study has several strengths and limitations. The main strength of this study is the use of mechanical testing to assess the effect of blueberry polyphenols on fracture risk in an animal model of post-menopausal bone loss, giving our results more direct relevance to the clinical endpoint of osteoporotic fracture than surrogate measures like BMD. One limitation of our study was the lack of sham operated animals to assess effect size. We chose not to include sham operated controls because our goal was to assess the effects of blueberry polyphenols in an osteoporotic model. Another limitation is the absence of baseline PIXImus scans, though, as discussed above, interference from surrounding bones prevented accurate scans in live animals.

In conclusion, our study of blueberry polyphenols in OVX rats failed to demonstrate benefits to bone as measured by BMD and three-point bending. Our results, coupled with those in other studies of polyphenols and bone fragility, do not give a clear picture as to how polyphenols may impact fracture risk. The paucity of available studies and heterogeneity of results point to the need for additional research in this area to help clarify the potential for polyphenols to mitigate postmenopausal bone loss and fragility fractures.

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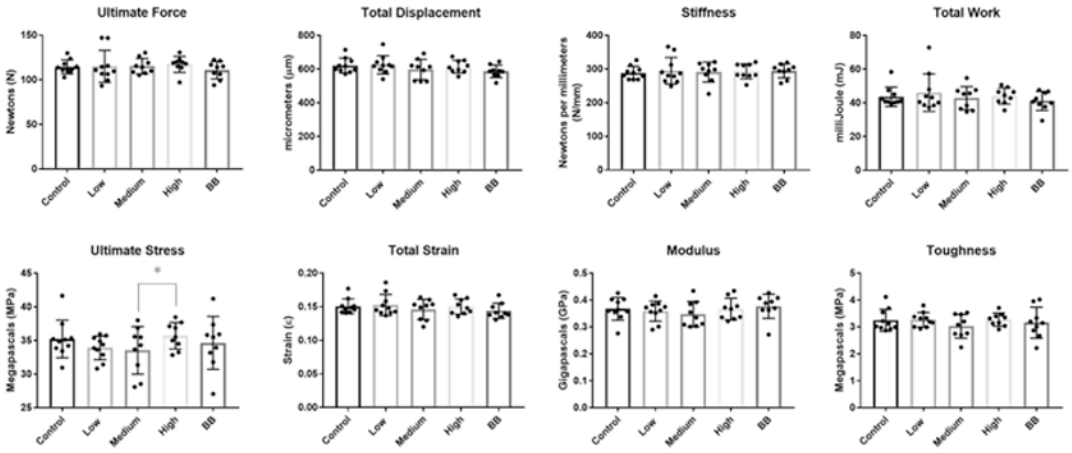

Figure 1 –.

Structure-level (A) and material-level (B) bone mechanical properties. Data shown as mean ± SD with individual animal data shown (dots). Results were analyzed using one-way ANOVA with Tukey's HSD post hoc test ($\alpha = 0.05$); statistically different results are indicated with (*). BB = lyophilized blueberry group

Table 1 –

Bone parameters measured by PIXImus and micro-CT in rats after 90d gavage with blueberry polyphenols.

	Control	Low	Medium	High	BB	*p*-value
Femur (PIXImus)						
BMC (g)	0.413 ± 0.031	0.434 ± 0.069	0.429 ± 0.038	0.434 ± 0.040	0.418 ± 0.024	0.75
Total Bone Area (cm^2)	2.14 ± 0.10	2.19 ± 0.15	2.15 ± 0.10	2.15 ± 0.09	2.10 ± 0.10	0.57
BMD (g/cm^2)	0.192 ± 0.009	0.198 ± 0.019	0.199 ± 0.012	0.202 ± 0.012	0.199 ± 0.007	0.54
Mid-diaphysis of femur (µCT)						
B.Ar (mm^2)	5.87 ± 0.40	5.89 ± 0.75	5.90 ± 0.43	5.82 ± 0.40	5.69 ± 0.40	0.81
Ct.Th. (mm)	0.596 ± 0.030	0.575 ± 0.032	0.574 ± 0.034	0.584 ± 0.040	0.563 ± 0.035	0.32
Tibia (PIXImus)						
BMC (g)	0.349 ± 0.024	0.374 ± 0.045	0.369 ± 0.031	0.378 ± 0.044	0.357 ± 0.020	0.34
Total Bone Area (cm^2)	2.18 ± 0.12	2.27 ± 0.10	2.21 ± 0.09	2.25 ± 0.14	2.17 ± 0.04	0.22
BMD (g/cm^2)	0.160 ± 0.005	0.165 ± 0.013	0.166 ± 0.008	0.167 ± 0.010	0.164 ± 0.006	0.45
L1-L4 Vertebrae (PIXImus)						
BMC (g)	0.430 ± 0.037	0.430 ± 0.053	0.438 ± 0.049	0.464 ± 0.069	0.425 ± 0.032	0.46
Total Bone Area (cm^2)	2.52 ± 0.16	2.52 ± 0.11	2.53 ± 0.15	2.56 ± 0.19	2.49 ± 0.10	0.88
BMD (g/cm^2)	0.171 ± 0.01	0.170 ± 0.015	0.173 ± 0.01	0.180 ± 0.014	0.171 ± 0.009	0.33

Data shown as mean ± SD. No significant differences were found between treatment groups for any parameters measured.

BMC = bone mineral content; BMD = bone mineral density; B.Ar = bone area; Ct.Th. = cortical thickness; BB = lyophilized blueberry group