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Reduced gravitational loading does not account for the skeletal effect of botulinum toxin-induced muscle inhibition suggesting a direct effect of muscle on bone

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Abstract

Intramuscular injection of botulinum toxin (botox) into rodent hindlimbs has developed as a useful model for exploring muscle–bone interactions. Botox-induced muscle inhibition rapidly induces muscle atrophy and subsequent bone loss, with the latter hypothesized to result from reduced muscular loading of the skeleton. However, botox-induced muscle inhibition also reduces gravitational loading (as evident by reduced ground reaction forces during gait) which may account for its negative skeletal effects. The aim of this study was to investigate the skeletal effect of botox-induced muscle inhibition in cage control and tail suspended mice, with tail suspension being used to control for the reduced gravitational loading associated with botox. Female C57BL/6J mice were injected unilaterally with botox and contralaterally with vehicle, and subsequently exposed to tail suspension or normal cage activities for 6 weeks. Botox-induced muscle inhibition combined with tail suspension had the largest detrimental effect on the skeleton, causing the least gains in midshaft tibial bone mass, cortical area and cortical thickness, greatest gains in midshaft tibial medullary area, and lowest proximal tibial trabecular bone volume fraction. These data indicate botox-induced muscle inhibition has skeletal effects over and above any effect it has in altering gravitational loading, suggesting that muscle has a direct effect on bone. This effect may be relevant in the development of strategies targeting musculoskeletal health.

Keywords

Botox; Mechanical loading; Muscle–bone interaction; Myokines; Tail suspension

Introduction

Skeletal tissue adapts to its mechanical environment by transducing mechanical stimuli into a cellular response via a process referred to as mechanotransduction [1]. The mechanical

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stimulus driving mechanotransduction is believed to derive from two primary sources—exogenous gravitational forces and endogenous muscle forces. Debate persists as to the relative roles of these two forces in mechanotransduction and their subsequent contributions to skeletal health [2–4]. In particular, the contribution of muscle forces to skeletal health has proven difficult to elicit as muscle and bone are inextricably linked genetically, mechanically, and molecularly.

Focusing on the mechanical link between muscle and bone, muscle-derived forces have been suggested to be both causative and protective of bone loading. Muscles attach close to axes of motion and thereby have small lever arms. They consequently need to generate and transmit high forces to the skeleton in order to produce a desired torque at the end of a lever (i.e. bone). It has subsequently been proposed that muscle-derived forces are the primary source of mechanical loading for bone [5,6], providing not only peak loads that generate the highest bone strains, but also low-magnitude high-frequency stimuli to which bone tissue may also respond [7].

It has also been hypothesized and modeled that muscle is protective of bone loading [8,9]. During impact loading, muscle is believed to act as an active shock absorber helping to attenuate impact loads as they are transmitted proximally along the kinetic chain. When muscles are dysfunctional (weakened, fatigued, or altered in their activation patterns) their ability to absorb loads becomes compromised, potentially leading to increased loading on the skeleton. For instance, laboratory-based studies have shown that muscle fatigue increases bone loading, as indicated by elevated bone strain magnitudes and rates [10–13]. Similarly, cross-sectional and prospective clinical studies report that susceptibility to mechanical overload-induced skeletal injury (i.e. stress fracture) is heightened in individuals with reduced indices of muscle performance [14–18].

A novel means of exploring the muscle–bone interaction has been to study the skeletal effects of intramuscular botulinum toxin (botox) injection. Botox blocks neuromuscular transmission by inhibiting the release of acetylcholine leading to locally reduced muscle activity, and resultant muscle atrophy and reduced strength. Previous studies have consistently demonstrated negative skeletal effects of botox-induced muscle inhibition in rodent models [19–32]. However, botox injected into the hindlimb muscles of rodents also reduces exogenous gravitational loading, as indicated by reduced vertical ground reaction forces (GRFs) during gait [27]. It remains unclear whether the reduction in GRFs associated with botox accounts for its skeletal effects. Manske et al. [25] contributed to this question by exploring the skeletal effects of combined Achilles tenotomy and botox-induced muscle inhibition; however, tenotomy incompletely controls for the effects of exogenous gravitational forces as partial weight bearing remains possible.

The aim of the current study was to investigate whether reduced gravitational loading accounts for the skeletal effects of botox-induced muscle inhibition by exploring the effects of botox in cage control and tail suspended mice. Tail suspension effectively removes exogenous loading of the hindlimbs (i.e. GRFs) such that all skeletal loading in the hindlimbs of tail suspended animals is generated endogenously by muscle. If the skeletal changes occurring following botox injection are due to the associated decrease in GRFs there would be no difference between muscle intact and botox inhibited hindlimbs in tail suspended animals. Conversely, if botox-induced muscle inhibition directly influences bone, skeletal status in muscle intact and inhibited hindlimbs of tail suspended animals would differ.

Methods

Animals

Forty virgin female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were acclimated until 14 weeks of age. Animals were maintained under standardized environmental conditions at all times with ad libitum access to standard mouse chow and water. Procedures were performed with approval from the Institutional Animal Care and Use Committee of Indiana University.

Muscle intervention

The right hindlimb of each animal was injected at baseline with *Clostridium botulinum* type A neurotoxin (BOTOX®; Allergan, Inc. Irvine, CA) (botox group). On the day of use, 100 U of botox was reconstituted in 4 ml of 0.9% sterile saline to create a solution with 0.025 U/μl. Animals were anesthetized using inhalation anesthesia, and the right quadriceps, hamstring, gastrocnemius and tibialis anterior muscles were injected with 20 μl (5 μl/muscle group) using a 25 μl Hamilton syringe equipped with a 30 G needle (Hamilton Co., Reno, NV). The left quadriceps, hamstring, gastrocnemius and tibialis anterior muscles were injected with an equivalent volume of 0.9% sterile saline (vehicle group) and served as internal controls.

Gravitational intervention

Immediately following muscle intervention, animals were randomly divided into two activity groups: 1) cage control and 2) tail suspended. The cage control group was allowed normal cage activities throughout the study, whereas the tail suspended group was tail suspended continuously for 6 weeks as previously described [33]. Half of a metal paper clip was made into a U-shape and its open end attached to the sides of the mouse tail with superglue. The rounded end of the paperclip was attached to a swivel and hung from an overhead wire. The wire height was adjusted to maintain the mice at 30° of head down tilt so that the hindlimbs but not forelimbs were elevated above the cage floor. The swivel allowed animal pivoting and slid freely on the wire to permit side-to-side movements. The cage floor was sparsely lined with bedding to absorb excretions. Suspended animals were floor fed.

In vivo peripheral quantitative computed tomography

In vivo skeletal and muscle assessments were performed under inhalation anesthesia at baseline and following 6 week intervention using a peripheral quantitative computed tomography (pQCT) machine equipped with software version 6.20C (Stratec XCT Research SA+, Stratec Medizintechnik GmbH, Pforzheim, Germany). Following a scout scan, a transverse midshaft scan was taken of each tibia using a 70 μm voxel size. This voxel size is relatively large compared to the cortical thickness of the mouse tibial midshaft increasing the potential for partial volume effects. However, good agreement has previously been shown between pQCT, micro-computerized tomography (μCT) and histological measures of cortical bone properties in mice [34].

Bone properties were obtained by placing a region of interest around the tibia and assessing using cortical mode 1 with a threshold of 400 mg/cm^3. Total bone mineral content (BMC, mg/cm), total bone area (Tt.Ar, mm^2), cortical area (Ct.Ar, mm^2), cortical thickness (Ct.Th, mm) and polar moment of inertia (I_P, mm^4) were recorded, and medullary area (Me.Ar, mm^2) derived as Tt.Ar minus Ct.Ar. Muscle cross-sectional area (mCSA, cm^2) was assessed by placing a region of interest around the entire leg (including the posterior, lateral and anterior muscle compartments), and using contour mode 3 (threshold, −100 mg/cm^3) to locate the skin surface and peel mode 2 (threshold, 40 mg/cm^3) to locate the subcutaneous fat–muscle boundary. A 3×3 kernel filter to filter all voxels between −500 and 500 mg/cm^3

followed by a 5×5 kernel filter to filter all voxels between −500 and 300 mg/cm^3 (F03F05 filter) was used to remove noise. All in vivo pQCT measures were expressed as percent change from baseline ([final – baseline]/baseline×100).

Ex vivo micro-computed tomography

Animals were euthanized following 6 week intervention (animal age=20 weeks), and the right and left tibias dissected free and placed in 10% neutral buffered formalin for 48 h before being stored in 70% ethanol. A desktop μCT machine (SkyScan 1172 high-resolution μCT; SkyScan, Kontich, Belgium) scanning with a source voltage of 59 kV and 11.8 μm isotropic voxel size was used to assess cortical bone properties at the midshaft tibia and trabecular bone properties within the proximal tibial metaphysis. For cortical bone properties, a single transverse midshaft tibia slice was imported into ImageJ v1.45s (National Institutes of Health, Bethesda, MD) and analyzed using the plugin BoneJ v1.3.3 [35] to acquire Tt.Ar (mm^2), Ct.Ar (mm^2), Me.Ar (mm^2), Ct.Th (μm) and I_P (mm^4). BMC (mg/cm) was obtained by exploiting the linear relationship between Hounsfield Units and known densities from calcium hydroxyapatite standards scanned with the same parameters as our bone samples. For trabecular bone properties, a 1 mmthick cross-sectional region was analyzed beginning 0.5 mm distal to the proximal tibial growth plate. The volume for analysis was within the trabecular compartment, and excluded cortical and subcortical bone. Trabecular bone volume fraction (bone volume [BV]/total volume [TV], %), number (Tb.N, mm^{-1}), thickness (Tb.Th, mm) and separation (Tb.Sp, mm) were acquired.

Histomorphometry

Calcein (30 mg/kg; Sigma Chemical Co., St. Louis, MO) and alizarin (50 mg/kg; Sigma Chemical Co., St. Louis, MO) injections were given 11 and 4 days prior to euthanasia to permit determination of bone formation rates. The tibias were embedded undecalcified in 99% methyl-methacrylate with 3% dibutyl phthalate (Sigma-Aldrich, St. Louis, MO). Transverse thick (40–50 μm) sections were removed from the tibial midshaft using a diamond-embedded wire saw (Histo-saw; Delaware Diamond Knives), and mounted unstained to assess periosteal bone formation rates. Frontal plane thin (4 μm) sections of the proximal tibia were taken using a microtome (Reichert-Jung 2050; Reichert-Jung, Heidelberg, Germany), and mounted either unstained to enable determination of trabecular bone formation rate or stained with tartrate-resistant acid phosphatase and counterstained with hematoxylin (Sigma-Aldrich, Kit #387A-1KT, St. Louis, MO) to allow identification of trabecular osteoclasts.

Sections were montaged using Image-Pro Plus (Version 7.0; Media Cybernetics, Inc., Bethesda, MD) on a Leica DMI6000 inverted microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) and stored digitally. Dynamic parameters were measured from the unstained midshaft and proximal tibia sections, and included single-label perimeter (sL.Pm), double-label area (dL.Ar) and perimeter (dL.Pm), and interlabel width (Ir.L.Wi). The following were derived from these primary data: mineralizing surface (MS/BS=[1/2sL.Pm+dL.Pm]/B.Pm, %), mineral apposition rate (MAR=mean Ir.L.Wi/7 days, μm/day), and bone formation rate (BFR/BS=MAR×MS/BS×3.65, μm^3/μm^2/yr). The region of interest within the proximal tibia consisted of a 1 mm^2 box positioned 1.0 mm distal from the growth plate within the secondary spongiosa. Bone resorption was determined from stained sections of the proximal tibia by counting the number of bone-adherent, multinucleate, tartrate-resistant acid phosphatase positive cells (osteoclasts) within 1 mm^2 of the secondary spongiosa and normalizing to bone surface (Oc.N/BS, N/mm).

Statistics

Analyses were performed with the IBMSPSS Statistics (version 19.0; SPSS Inc., Chicago, IL) software, and were two-tailed with a level of significance set at 0.05 (unless otherwise specified). Two-way one-repeated-measure analyses of variance (ANOVA) were used for initial comparisons, with muscle group (vehicle vs. botox) being the within-animal and gravitational group (cage control vs. tail suspended) being the between-animal independent variables. In the advent of a non-significant ANOVA interaction, main effects for each independent variable were explored. Significant ANOVA interactions were explored using 4 simple effect tests to assess for the effect of gravitational intervention in each muscle group (unpaired t-tests) and muscle intervention in each gravitational group (paired t-tests), with a Bonferroni correction to the level of significance used to maintain the familywise error rate (α = 0.05/4=0.0125).

Results

Animal characterization

Gravitational intervention had no effect on change in body mass across the 6 week intervention period (cage control=2.9±3.6 g vs. tail suspended=2.5±6.1 g, p=0.80 [*unpaired t-test*]). There was a significant gravitational × muscle group interaction on change in mCSA (p<0.01), with botox injection having a lesser effect on change in mCSA in tail suspended animals compared to cage control animals (−19.2% [95%CI, −24.0 to −14.3%; p<0.001] vs. −29.0% [95%CI, −34.0 to −24.0%; p<0.001], respectively) (Fig. 1). Despite the lesser effect of botox injection in tail suspended animals, the combined interventions had the greatest effect on change in mCSA indicating that botox injection had some effects over-and-above tail suspension.

Cortical bone effects of gravitational and muscle intervention

Longitudinal in vivo pQCT measures of the midshaft tibia revealed significant gravitational × muscle group interactions on change in BMC and I_P (all p<0.01). The interaction on change in BMC resulted from botox injection having a lesser effect in tail suspended animals compared to cage control animals (−6.0% [95%CI, −8.4 to −3.5%; p<0.001] vs. −12.7% [95%CI, −15.7 to −9.7%; p<0.001], respectively) (Fig. 2A). However, the combined introduction of the interventions had the greatest effect on change in BMC indicating that botox injection had some effects over-and-above tail suspension. For I_P, combined intervention removed the statistical effect of botox injection in tail suspended animals (p>0.0125) (Fig. 2F).

There were no gravitational × muscle group interactions on change in any other cortical bone measure indicating botox injection had similar effects in both tail suspended and cage control animals (all p=0.07 to 0.48). There was no main effect of tail suspension on change in Tt.Ar (p=0.46) (Fig. 2B); however, tail suspension increased gain in Me.Ar (22.7% [95%CI, 15.4 to 29.9%]) (Fig. 2D), and reduced gains in Ct.Ar (−8.1% [95%CI, −10.4 to −5.9%]) (Fig. 2C) and Ct.Th (−10.8% [95%CI, −13.3 to −8.2%]) (Fig. 2E) (all p<0.001). Botox injection increased gain in Me.Ar (10.0% [95%CI, 4.2 to 15.6%]) (Fig. 2D), and reduced gains in Tt.Ar (−2.8% [95%CI, −4.7 to −0.9%]) (Fig. 2B), Ct.Ar (−7.0% [95%CI, −9.0 to −4.9%]) (Fig. 2C) and Ct.Th (−7.1% [95%CI, −9.0 to −5.2%]) (Fig. 2E) (all $p \leq$ 0.03).

Ex vivo cross-sectional μCT measures of the midshaft tibia confirmed the independent main effects of gravitational and muscle intervention on cortical bone mass and structural properties (Fig. 3 and Table 1). Bones from tail suspended animals had 16.4% less BMC (−0.13 mg/cm [95%CI, −0.15 to −0.10 mg/cm]) after the intervention period, with 14.5%

less Ct.Ar (−0.09 mm^2 [95%CI, −0.11 to −0.07 mm^2]) and 28.4% less Ct.Th (−29 μm [95%CI, −35 to −22 μm]) due to 25.5% greater Me.Ar (0.09 mm^2 [95%CI, 0.06 to 0.12 mm^2]) (all $p<0.001$). Botox injection accentuated these changes such that combined intervention had the greatest impact on cortical bone properties. Bones from botox-injected hindlimbs had 9.3% less BMC (−0.07 mg/cm [95%CI, −0.08 to −0.06 mg/cm]), with 8.2% less Ct.Ar (−0.05 mm^2 [95%CI, −0.06 to −0.04 mm^2]) and 10.9% less Ct.Th (−10 μm [95%CI, −13 to −7 μm]) due to 7.5% greater Me.Ar (0.03 mm^2 [95%CI, 0.02 to 0.04 mm^2]) (all $p<0.001$).

The altered cortical bone properties with gravitational and muscle intervention were reflected in histomorphometric indices of periosteal bone formation. There was a significant gravitational × muscle group interaction on periosteal BFR/BS ($p<0.01$),with the negative statistical effect of botox injection in cage control animals ($p=0.002$) being absent in tail suspended animals. ($p=0.16$) (Fig. 4A). There were independent negative main effects for both tail suspension and botox injection on MS/BS and MAR (all $p\leq0.02$; *data not shown*) indicating that botox injection retained some of its effects on cortical bone formation in tail suspended animals.

Trabecular bone effects of gravitational and muscle loading intervention

There were significant (all $p\leq0.02$) gravitational × muscle group interactions on BV/TV, Tb.Th and Tb.N, and a main effect ($p<0.001$) for tail suspension on Tb.Sp (Fig. 5). Focusing on BV/TV as the principal measure of trabecular bone status, combined tail suspension and botox injection had a lesser effect in tail suspended animals compared to cage control animals (−0.7% [95%CI, −1.2 to −0.3%; $p<0.01$] vs. −2.9% [95%CI, −3.6 to −2.2%; $p<0.001$], respectively) (Fig. 5B). However, the combined interventions had the greatest effect on BV/TV indicating that botox injection had some trabecular bone effects over-and-above tail suspension.

The alterations in trabecular architecture with gravitational and muscle intervention were associated with changes in trabecular bone formation (Fig. 4B). There was no gravitational × muscle loading interaction on trabecular BFR/BS (all $p=0.40$ to 0.79); however, tail suspension and botox injection independently decreased trabecular BFR/BS by 41% (−92.9 $\mu m^3/\mu m^2$/yr [95%CI, −140.1 to −45.6 $\mu m^3/\mu m^2$/yr; $p\leq0.001$]) and 25% (−52.4 $\mu m^3/\mu m^2$/yr [95%CI, −96.0 to −8.9 $\mu m^3/\mu m^2$/yr; $p=0.02$]), respectively (Fig. 4B). There were no tail suspension or botox injection effects on Oc.N/BS (all $p\geq0.45$) (Fig. 4C).

Discussion

The results of this study indicate that the skeletal effects of botox-induced muscle inhibition are not solely due to a reduction in gravitational loading. The removal of gravitational loading of the hindlimbs via tail suspension induced a loss of bone mass as a result of decreased periosteal bone formation and increased endosteal bone resorption. The increase in endosteal bone resorption was evident by a significant increase in Me.Ar, and contributed to a reduction in Ct.Ar and Ct.Th. Superimposing botox-induced muscle inhibition on tail suspension exacerbated these skeletal changes, with combined gravitational and muscle intervention having the largest detrimental effect on the skeleton. Combined intervention led to the least gains in midshaft tibial BMC, Ct.Ar and Ct.Th, greatest gains in Me.Ar, and lowest proximal tibial BV/TV. The inferior bone health in botox injected hindlimbs of tail suspended animals indicates that botox-induced muscle inhibition has skeletal effects over-and-above those associated with altered gravitational loading. These data suggest that muscle has a direct effect on bone.

The negative skeletal effects of botox observed in cage control animals are consistent with previous studies exploring the skeletal effects of botox-induced muscle inhibition [19–32], with the current study furthering the knowledge base by demonstrating that the skeletal changes associated with botox injection are not simply due to a reduction in exogenous gravitational loading. The inability of reduced gravitational loading to account for the skeletal changes associated with botox injection supports the observations of Manske et al. [25] who demonstrated combined Achilles tenotomy and botox-induced muscle inhibition induced greater skeletal changes compared to botox alone. However, the latter study was limited by the incomplete control of exogenous gravitational forces due to variable healing of the Achilles tendon and the ability of animals to partially bear weight following tenotomy.

Combined tail suspension and botox-induced muscle inhibition had effects on a number of bone properties that were less than additive of the individual effects of the interventions. The less than additive effects were evident by the presence of statistical interactions, and were expected because of the dependent relationship between exogenous gravitational forces and endogenous muscle forces. A reduction in gravitational forces subsequently reduces the resistance against which muscles need to contract to maintain posture and produce joint motion. Thus, a reduction in gravitational forces (i.e. via tail suspension) also reduces muscle forces. This was captured in the current study by the reduced gain in mCSA in vehicle treated hindlimbs of tail suspended animals. Similarly, a reduction in muscle forces (i.e. via botox injection) results in a reduction in gravitational forces, as previously reported [27]. As the individual interventions utilized in the current study influenced a variable amount of the other, the combined effects of botox-induced muscle inhibition and tail suspension were less than additive on some measures.

The results of this study indicate that either botox injection or tail suspension may be used as a bone loss model, with the interventions individually inducing a loss of BMC over the 6 week intervention period. The benefits of botox include its ease of implementation and provision of a contralateral internal control site, with the potential caveat being the possibility of contralateral skeletal effects of botox [32]. Tail suspension had a greater skeletal effect than botox injection in the current study causing a greater loss of midshaft tibial BMC and lower proximal tibial BV/TV. The greater loss of midshaft tibial BMC with tail suspension was coupled with more reduced periosteal BFR/BS and greater gains in Me.Ar compared to botox-injection, with the greater gains in Me.Ar suggesting more heightened bone resorption with tail suspension. These observations appear to disagree with Gross and colleagues [19,29,32] who suggested that botox-induced muscle inhibition has a greater skeletal effect than tail suspension, and that the skeletal effect of botox is predominantly due to heightened bone resorption. The most likely explanation for the seemingly contrasting finding in our study is the relatively long duration of the intervention period. Botox-induced muscle inhibition induces rapid bone degradation, with osteoclast numbers more than doubling within 5 days following injection [19] leading to a maximal reduction in bone mass within the initial 2–4 weeks [19,23,24,26,27,29]. Beyond this early period, both muscle and bone begin to recover as the effects of botox reverse [23,24,26,27,29]. The reversal of botox effects and our relatively late analysis time point may explain the lack of a significant finding on our primary indicator of bone resorption (Oc.N/BS within the proximal tibia). Similarly, the reversible effects of botox intervention relative to more persistent exposure to tail suspension intervention may explain the greater skeletal effect of the latter in the current study.

The current study supports a direct effect of muscle on bone, with muscle-derived mechanical loading of the skeleton being the likely candidate mechanism. However, a potential molecular link between muscle and bone exists which may also contribute to the

skeletal effects of muscle. In particular, muscle secretes a variety of cytokines and growth factors collectively referred to as myokines which may exert autocrine, paracrine or endocrine effects [36]. A number of these myokines have known or putative roles on bone metabolism, including insulin-like growth factor-1, fibroblast growth factor-2 and growth differentiation factor-8 (also known as myostatin) [37–39]. The potential for a molecular link between muscle and bone is of great interest as it may lead to the development of novel therapies that target both sarcopenia and osteoporosis.

The current data convincingly demonstrate that botox-induced muscle inhibition has an effect on bone beyond its effect of reducing gravitational loading. This suggests that muscle has a direct effect on bone; however, the data need to be interpreted in light of some study limitations. The current study was not designed to assess the mechanism for how muscle influences bone. The candidate mechanism is the mechanical link between muscle and bone, but muscle forces on the bone were not assessed and non-mechanical mechanisms cannot be excluded. For instance, if the secretion of osteogenic myokines occurs in relation to muscle size, molecular mechanisms may explain the observed predictive relationships between change in mCSA and change in bone properties (see Supplemental Fig. 1). It is also possible that the skeletal effects of botox are independent of its effects on muscle. For instance, botox introduced intramuscularly may diffuse locally to directly influence bone cells or travel via retrograde and anterograde axonal transport to influence bone-relevant distant sites.

A further limitation in the current study was the performance of outcome measures 6 weeks following a single botox dose. The mCSA effects of botox in mice begin to reverse at 3–4 weeks post-injection [26,27,29], with recovery from the functional deficits initiating even earlier [27,32]. It is possible that partial recovery in botox injected hindlimbs influenced data in the current study. Similarly, the data may have been influenced by early tail suspension-induced changes in body mass that subsequently recovered. However, we do not believe that these limitations altered the conclusions drawn. In particular, partial muscle recovery and early changes in body mass are unable to account for the detrimental skeletal effects of botox observed after 6 week intervention within tail suspended animals.

In conclusion, the current study demonstrates that the skeletal effects of botox-induced muscle inhibition are not solely due to a reduction in gravitational loading. Combined introduction of botox-induced muscle inhibition and tail suspension resulted in the greatest detrimental effect on the skeleton indicating that botox-induced muscle inhibition had skeletal effects over-and-above those associated with altered gravitational loading. These data suggest that muscle has a direct effect on bone. This direct effect may be relevant in the development of novel strategies targeting musculoskeletal health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Duncan RL, Turner CH. Mechanotransduction and the functional response of bone to mechanical strain. Calcif Tissue Int. 1995; 57:344–58. [PubMed: 8564797]
2. Judex S, Carlson KJ. Is bone’s response to mechanical signals dominated by gravitational loading? Med Sci Sports Exerc. 2009; 41:2037–43. [PubMed: 19812513]

3. Kohrt WM, Barry DW, Schwartz RS. Muscle forces or gravity: what predominates mechanical loading on bone? Med Sci Sports Exerc. 2009; 41:2050–5. [PubMed: 19812511]
4. Robling AG. Is bone's response to mechanical signals dominated by muscle forces? Med Sci Sports Exerc. 2009; 41:2044–9. [PubMed: 19812512]
5. Burr DB. Muscle strength, bone mass, and age-related bone loss. J Bone Miner Res. 1997; 12:1547–51. [PubMed: 9333114]
6. Frost HM. On our age-related bone loss: insights from a new paradigm. J Bone Miner Res. 1997; 12:1539–46. [PubMed: 9333113]
7. Judex S, Rubin CT. Is bone formation induced by high-frequency mechanical signals modulated by muscle activity? J Musculoskelet Neuronal Interact. 2010; 10:3–11. [PubMed: 20190375]
8. Donahue, SW. The role of muscular force and fatigue in stress fractures. In: Burr, DB.; Milgrom, C., editors. Musculoskeletal fatigue and stress fractures. Bota Raton, Florida, USA: CRC Press; 2001. p. 131-49.
9. Scott SH, Winter DA. Internal forces at chronic running injury sites. Med Sci Sports Exerc. 1990; 22:357–69. [PubMed: 2381304]
10. Arndt A, Ekenman I, Westblad P, Lundberg A. Effects of fatigue and load variation on metatarsal deformation measured in vivo during barefoot walking. J Biomech. 2002; 35:621–8. [PubMed: 11955501]
11. Fyhrie DP, Milgrom C, Hoshaw SJ, Simkin A, Dar S, Drumb D, et al. Effect of fatiguing exercise on longitudinal bone strain as related to stress fracture in humans. Ann Biomed Eng. 1998; 26:660–5. [PubMed: 9662157]
12. Milgrom C, Radeva-Petrova DR, Finestone A, Nyska M, Mendelson S, Benjuya N, et al. The effect of muscle fatigue on in vivo tibial strains. J Biomech. 2007; 40:845–50. [PubMed: 16682046]
13. Sharkey NA, Ferris L, Smith TS, Matthews DK. Strain and loading of the second metatarsal during heel-lift. J Bone Joint Surg Am. 1995; 77:1050–7. [PubMed: 7608227]
14. Armstrong DW III, Rue JPH, Wilckens JH, Frassica FJ. Stress fracture injury in young military men and women. Bone. 2004; 35:806–16. [PubMed: 15336620]
15. Beck TJ, Ruff CB, Shaffer RA, Betsinger K, Trone DW, Brodine SK. Stress fracture in military recruits: gender differences in muscle and bone susceptibility factors. Bone. 2000; 27:437–44. [PubMed: 10962357]
16. Bennell KL, Malcolm SA, Thomas SA, Reid SJ, Brukner PD, Ebeling PR, et al. Risk factors for stress fractures in track and field athletes: a twelve-month prospective study. Am J Sports Med. 1996; 24:810–8. [PubMed: 8947404]
17. Popp KL, Hughes JM, Smock AJ, Novotny SA, Stovitz SD, Koehler SM, et al. Bone geometry, strength, and muscle size in runners with a history of stress fracture. Med Sci Sports Exerc. 2009; 41:2145–50. [PubMed: 19915505]
18. Schnackenburg KE, Macdonald HM, Ferber R, Wiley JP, Boyd SK. Bone quality and muscle strength in female athletes with lower limb stress fractures. Med Sci Sports Exerc. 2011; 43:2110–9. [PubMed: 21552163]
19. Aliprantis AO, Stolina M, Kostenuik PJ, Poliachik SL, Warner SE, Bain SD, et al. Transient muscle paralysis degrades bone via rapid osteoclastogenesis. FASEB J. 2012; 26:1110–8. [PubMed: 22125315]
20. Ausk BJ, Huber P, Poliachik SL, Bain SD, Srinivasan S, Gross TS. Cortical bone resorption following muscle paralysis is spatially heterogeneous. Bone. 2012; 50:14–22. [PubMed: 21920486]
21. Bouvard B, Mabilleau G, Legrand E, Audran M, Chappard D. Micro and macroarchitectural changes at the tibia after botulinum toxin injection in the growing rat. Bone. 2012; 50:858–64. [PubMed: 22252044]
22. Chappard D, Chennebault A, Moreau M, Legrand E, Audran M, Basle MF. Texture analysis of X-ray radiographs is a more reliable descriptor of bone loss than mineral content in a rat model of localized disuse induced by the *Clostridium botulinum* toxin. Bone. 2001; 28:72–9. [PubMed: 11165945]

23. Grimston SK, Goldberg DB, Watkins M, Brodt MD, Silva MJ, Civitelli R. Connexin43 deficiency reduces the sensitivity of cortical bone to the effects of muscle paralysis. J Bone Miner Res. 2011; 26:2151–60. [PubMed: 21590735]
24. Grimston SK, Silva MJ, Civitelli R. Bone loss after temporarily induced muscle paralysis by botox is not fully recovered after 12 weeks. Ann N Y Acad Sci. 2007; 1116:444–60. [PubMed: 17584988]
25. Manske SL, Boyd SK, Zernicke RF. Muscle changes can account for bone loss after botulinum toxin injection. Calcif Tissue Int. 2010; 87:541–9. [PubMed: 20967431]
26. Manske SL, Boyd SK, Zernicke RF. Muscle and bone follow similar temporal patterns of recovery from muscle-induced disuse due to botulinum toxin injection. Bone. 2010; 46:24–31. [PubMed: 19853070]
27. Manske SL, Boyd SK, Zernicke RF. Vertical ground reaction forces diminish in mice after botulinum toxin injection. J Biomech. 2011; 44:637–43. [PubMed: 21186027]
28. Manske SL, Good CA, Zernicke RF, Boyd SK. High-frequency, low-magnitude vibration does not prevent bone loss resulting from muscle disuse in mice following botulinum toxin injection. PLoS One. 2012; 7:e36486. [PubMed: 22590551]
29. Poliachik SL, Bain SD, Threet D, Huber P, Gross TS. Transient muscle paralysis disrupts bone homeostasis by rapid degradation of bone morphology. Bone. 2010; 46:18–23. [PubMed: 19857614]
30. Sheng ZF, Ma YL, Tong D, Fang DY, Liang QC, Liu LH, et al. Strontium ranelate prevents bone loss in a rat model of localized muscle paralysis. Ann Biomed Eng. 2012; 40:657–65. [PubMed: 22237860]
31. Thomsen JS, Christensen LL, Vegger JB, Nyengaard JR, Bruel A. Loss of bone strength is dependent on skeletal site in disuse osteoporosis in rats. Calcif Tissue Int. 2012; 90:294–306. [PubMed: 22354132]
32. Warner SE, Sanford DA, Becker BA, Bain SD, Srinivasan S, Gross TS. Botox induced muscle paralysis rapidly degrades bone. Bone. 2006; 38:257–64. [PubMed: 16185943]
33. Warden SJ, Hassett SM, Bond JL, Rydberg J, Grogg JD, Hilles EL, et al. Psychotropic drugs have contrasting skeletal effects that are independent of their effects on physical activity levels. Bone. 2010; 46:985–92. [PubMed: 20060080]
34. Schmidt C, Priemel M, Kohler T, Weusten A, Muller R, Amling M, et al. Precision and accuracy of peripheral quantitative computed tomography (pQCT) in the mouse skeleton compared with histology and microcomputed tomography (microCT). J Bone Miner Res. 2003; 18:1486–96. [PubMed: 12929938]
35. Doube M, Klosowski MM, Arganda-Carreras I, Cordelieres FP, Dougherty RP, Jackson JS, et al. BoneJ: free and extensible bone image analysis in ImageJ. Bone. 2010; 47:1076–9. [PubMed: 20817052]
36. Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nat Rev Endocrinol. 2012; 8:457–65. [PubMed: 22473333]
37. Elkasrawy MN, Hamrick MW. Myostatin (GDF-8) as a key factor linking muscle mass and bone structure. J Musculoskelet Neuronal Interact. 2010; 10:56–63. [PubMed: 20190380]
38. Hamrick MW, McNeil PL, Patterson SL. Role of muscle-derived growth factors in bone formation. J Musculoskelet Neuronal Interact. 2010; 10:64–70. [PubMed: 20190381]
39. Hamrick MW. A role for myokines in muscle–bone interactions. Exerc Sport Sci Rev. 2011; 39:43–7. [PubMed: 21088601]

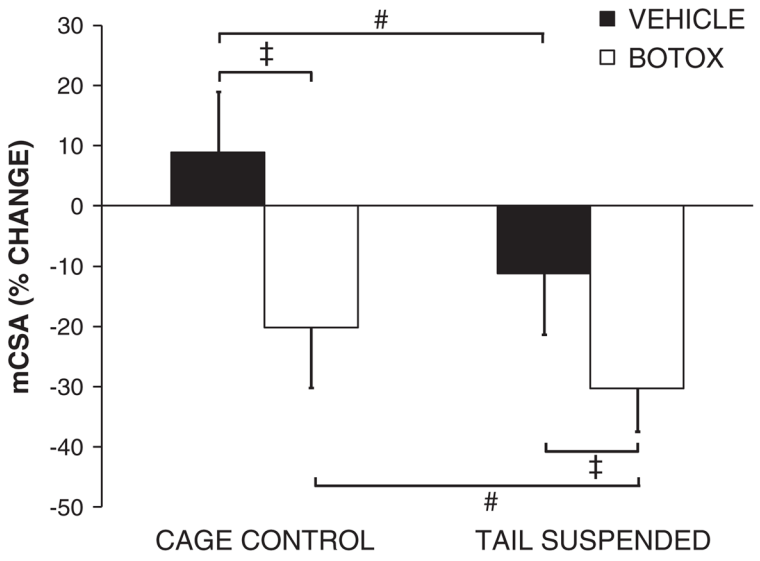

Fig. 1.

Effect of gravitational and muscle interventions on in vivo percent change in muscle cross-sectional area (mCSA) at the level of the midshaft tibia. There was a significant gravitational × muscle group interaction ($p<0.01$). ‡Botox reduced gains in mCSA in both cage control and tail suspended animals and #tail suspension reduced gains in mCSA in both vehicle and botox injected hindlimbs (all $p<0.0125$). Bars represent mean±SD.

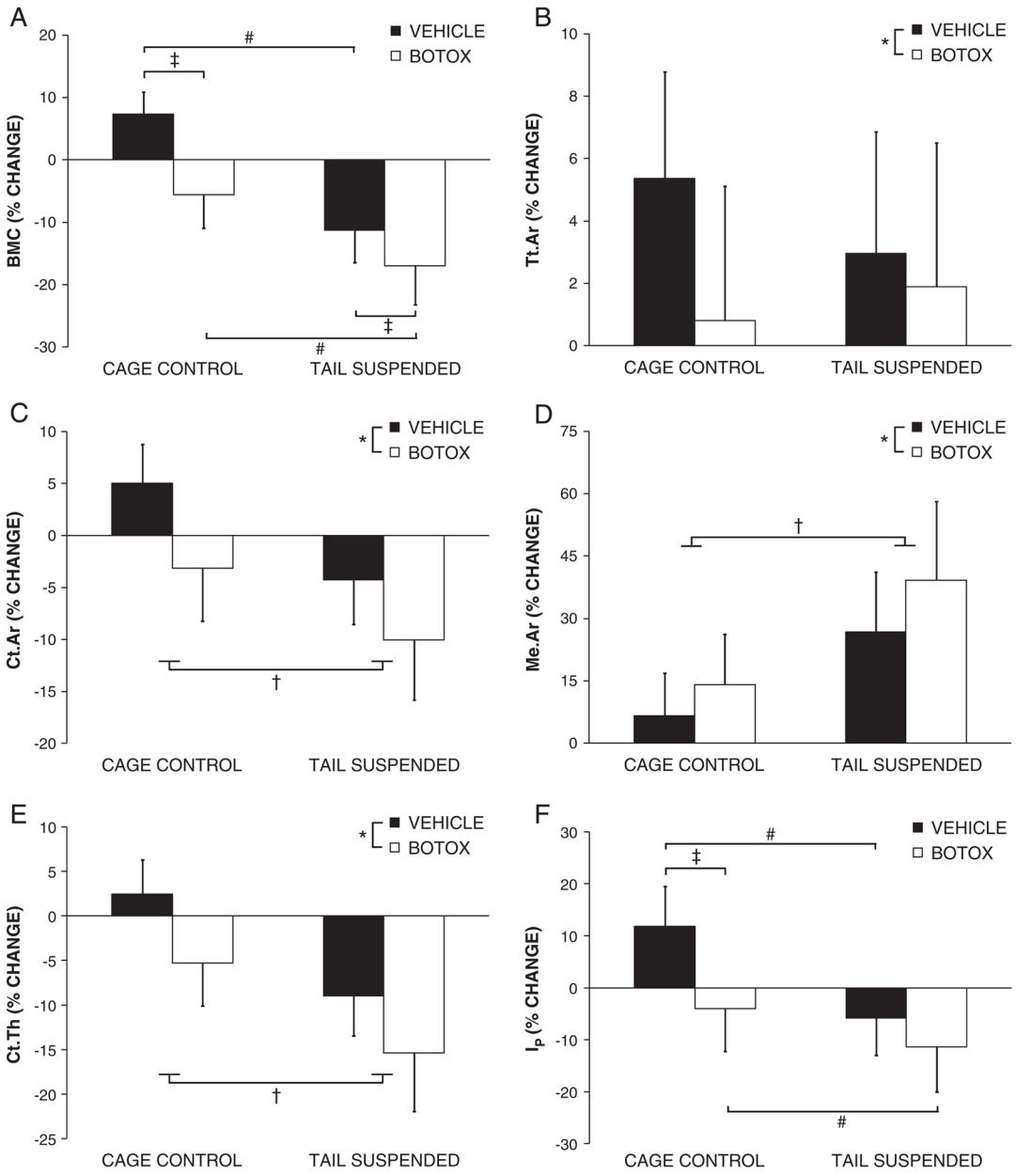

Fig. 2.

Effect of gravitational and muscle interventions on in vivo percent change in midshaft tibial: A) bone mineral content [BMC]; B) total area [Tt.Ar]; C) cortical area [Ct.Ar], D) medullary area [Me.Ar]; E) cortical thickness [Ct.Th], and; F) polar moment of inertia [I_P]. There were significant main effects for †tail suspension and *botox injection on Ct.Ar, Me.Ar and Ct.Th, and a significant main effect for *botox injection on Tt.Ar (all $p<0.05$). There were significant gravitational × muscle loading interactions on BMC and I_P (all $p<0.01$). ‡Botox reduced gains in BMC in both cage control and tail suspended animals, and I_P in cage control animals (all $p<0.0125$). #Tail suspension reduced gains in BMC and I_P in both vehicle and botox injected hindlimbs (all $p<0.0125$). Bars represent mean±SD.

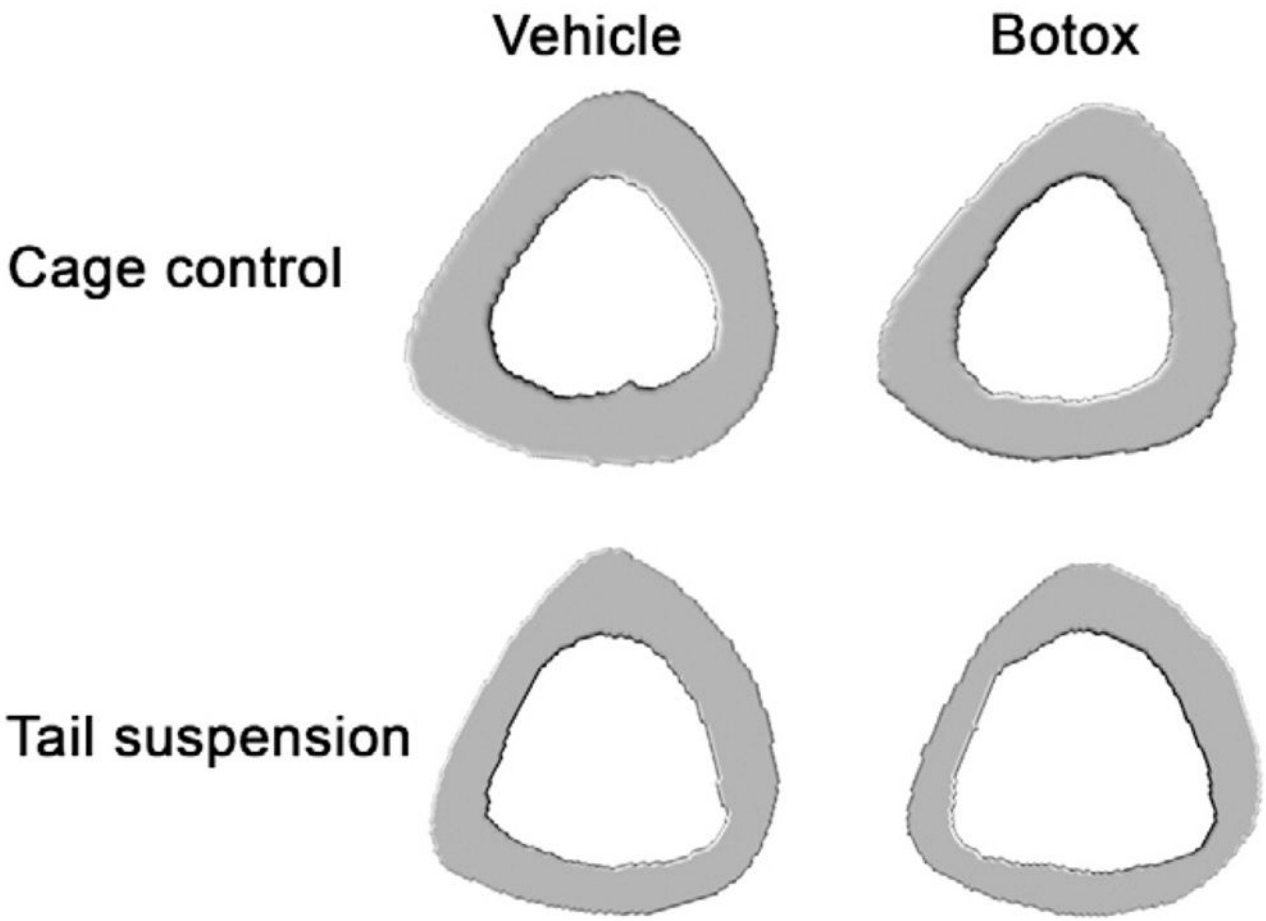

Fig. 3.

Effect of gravitational and muscle interventions on structure of the midshaft tibia, as shown in representative μCT cross-sectional images. Note the reduced cortical area and thickness with both tail suspension and botox intervention as a result of enlarged medullary area. These structural changes were exacerbated in bones exposed to combined tail suspension and botox intervention.

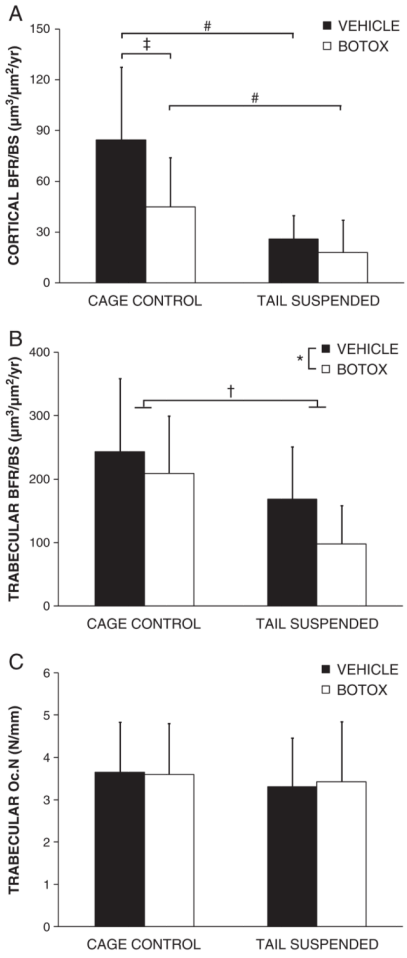

Fig. 4.

Effect of gravitational and muscle interventions on: A) midshaft tibial periosteal [cortical] bone formation rate [BFR/BS]; B) proximal tibial trabecular BFR/BS, and; C) proximal tibial trabecular osteoclast number [N.Oc/BS]. There was a significant gravitational × muscle loading interaction on midshaft tibial periosteal BFR/BS ($p<0.01$). ‡Botox reduced periosteal BFR/BS in cage control animals and $^{\#}$tail suspension reduced gains in periosteal BFR/BS in both vehicle and botox injected hindlimbs (all $p<0.0125$). There were significant main effects for †tail suspension and *botox injection on proximal tibial trabecular BFR/BS (all $p<0.05$). Bars represent mean±SD.

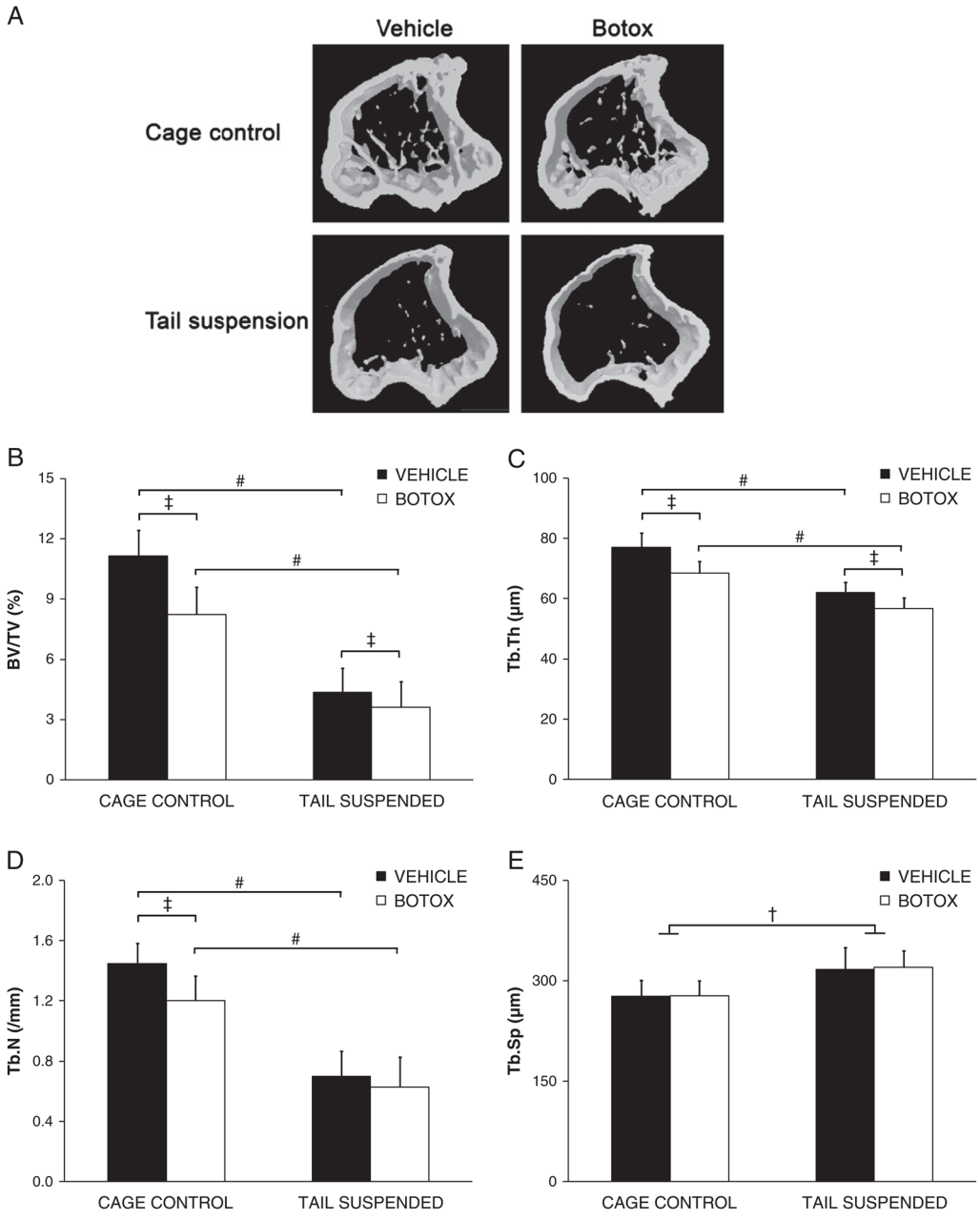

Fig. 5.

Effect of gravitational and muscle interventions on: A) representative [250 μm thick] axial plane three-dimensional reconstructions of trabecular architecture within the proximal tibia; B) trabecular bone volume fraction [BV/TV]; C) trabecular thickness [Tb.Th]; D) trabecular number [Tb.N], and; E) trabecular separation [Tb.Sp]. In A), note the reduced amount of trabecular bone with individual and combined introduction of botox and tail suspension. There were significant gravitational × muscle loading interactions on BV/TV, Tb.Th and Tb.N (all $p \leq 0.02$), and a significant main effect for tail suspension on Tb.Sp ($p < 0.001$). ‡Botox decreased BV/TV, Tb.Th and Tb.N in cage control animals, and BV/TV and Tb.Th in tail suspended animals (all $p < 0.0125$). #Tail suspension decreased BV/TV,

Tb.Th and Tb.N in both vehicle and botox injected hindlimbs (all $p<0.0125$). Bars in (B–E) represent mean±SD.

Table 1

Effect of gravitational and muscle interventions on ex vivo midshaft tibia cortical bone properties.[a]

Bone property[b]	Cage control		Tail suspended		Two-way ANOVA		
	Vehicle	Botox	Vehicle	Botox	Grav.x Muscle	Gravitational	Muscle
Ct.BMC (mg/cm)	8.09±0.39	7.31±0.39	6.74±0.49	6.14±0.53	NS	<0.001	<0.001
Tt.Ar (mm^2)	1.01±0.06	0.98±0.06	1.00±0.06	0.99±0.06	NS	NS	0.02
Ct.Ar (mm^2)	0.66±0.03	0.60±0.03	0.56±0.04	0.51±0.04	NS	<0.001	<0.001
Me.Ar (mm^2)	0.36±0.04	0.38±0.04	0.44±0.06	0.48±0.06	NS	<0.001	<0.001
Ct.Th (μm)	107±13	96±10	77±11	69±9	NS	<0.001	<0.001
I_P (mm^4)	0.15±0.02	0.13±0.02†	0.13±0.02‡	0.12±0.01†	0.04	_[c]	_[c]

[a] Data are mean±SD.

[b] Ct.BMC=cortical bone mineral content; Tt.Ar=total area; Ct.Ar=cortical area; Me.Ar=medullary area; Ct.Th=cortical thickness; Ip=polar moment of inertia.

[c] Gravitational and muscle group main effects were ignored in the presence of a significant gravitational × muscle group interaction.

† $p<0.0125$ vs. vehicle injected hindlimb within same gravitational group (paired *t*-test).

‡ $p<0.0125$ vs. vehicle injected hindlimb within cage control (unpaired *t*-test).