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Botox induced muscle paralysis rapidly degrades bone

Sarah E. Warner^a, David A. Sanford^a, Blair A. Becker^a, Steven D. Bain^b, Sundar Srinivasan^a, and Ted S. Gross^{a,*}

a Department of Orthopaedics and Sports Medicine, University of Washington, 325 Ninth Ave, Box 359798, Seattle, WA 98104-2499, USA

b SkeleTech, Inc., 22002 26th Ave. SE, Suite 104 Bothell, WA 98021, USA

Abstract

The means by which muscle function modulates bone homeostasis is poorly understood. To begin to address this issue, we have developed a novel murine model of unilateral transient hindlimb muscle paralysis using botulinum toxin A (Botox). Female C57BL/6 mice (16 weeks) received IM injections of either saline or Botox ($n = 10$ each) in both the quadriceps and calf muscles of the right hindleg. Gait dysfunction was assessed by multi-observer inventory, muscle alterations were determined by wet mass, and bone alterations were assessed by micro-CT imaging at the distal femur, proximal tibia, and tibia mid-diaphysis. Profound degradation of both muscle and bone was observed within 21 days despite significant restoration of weight bearing function by 14 days. The muscle mass of the injected quadriceps and calf muscles was diminished -47.3% and -59.7% , respectively, vs. saline mice (both $P < 0.001$). The ratio of bone volume to tissue volume (BV/TV) within the distal femoral epiphysis and proximal tibial metaphysis of Botox injected limbs was reduced -43.2% and -54.3% , respectively, while tibia cortical bone volume was reduced -14.6% (all $P < 0.001$). Comparison of the contralateral non-injected limbs indicated the presence of moderate systemic effects in the model that were most probably associated with diminished activity following muscle paralysis. Taken as a whole, the micro-CT data implied that trabecular and cortical bone loss was primarily achieved by bone resorption. These data confirm the decisive role of neuromuscular function in mediating bone homeostasis and establish a model with unique potential to explore the mechanisms underlying this relation. Given the rapidly expanding use of neuromuscular inhibitors for indications such as pain reduction, these data also raise the critical need to monitor bone loss in these patients.

Keywords

Bone loss; Bone resorption; Disuse; Micro-CT; Muscle atrophy; Muscle function

Introduction

The integral role that muscle serves to maintain bone health is poignantly revealed by pathologies in which muscle function is chronically altered [1,23]. In acute conditions that result in diminished or impaired muscle function, such as bedrest or spinal cord injury, the annualized percentage of bone loss can range between 5% and 25%, depending upon skeletal site and severity of impairment [20,22]. As a comparison, annual bone loss associated with menopause is typically reported at 2 to 3% [30]. However, the means by which muscle function enables bone homeostasis are poorly understood.

In a mechanical context, muscles provide the means by which skeletal loading is achieved and therefore influence bone directly by modulating its mechanical environment. Given the wide

* Corresponding author. Fax: +1 206 341 5611. E-mail address: tgross@u.washington.edu (T.S. Gross)..

variety of muscle attachments and the complexity of skeletal loading induced by ground reaction forces, it is not surprising that bone strains are highly heterogeneous [11]. The relatively large strains induced by activity are superimposed upon a background of low magnitude high frequency strains [9]. Bone is intensely sensitive to alterations in either component of its mechanical milieu [29,33].

Coincident regional alterations in muscle and bone properties also suggest an underlying relation between the tissues [10,27]. Muscle function is critical for fetal bone development [21,31], muscle and bone both demonstrate a decline in mass and strength with aging [7,36], and muscle degradation is proportional to bone loss in spinal cord injury patients [39]. Further, local muscle stimulation of patients with spinal cord injury locally mitigates a portion of paralysis induced bone loss [3]. However, evidence that mechanistically associates muscle function and bone morphology remains elusive.

Murine models have demonstrated unique potential for mechanistic exploration of muscle/bone interactions in vivo [19], but bone loss in current murine models of disuse arises due to a combination of diminished osteoblast activity and enhanced osteoclastic resorption [34]. This response is distinct from that observed in humans and other mammals in which diminished muscle function precipitates reduced bone mass primarily via aggressive bone resorption with minimal contribution from altered osteoblast function [24]. This modeling mismatch has limited in vivo mechanistic exploration of muscle/bone interactions. We have therefore developed a murine model in which the primary extensors of the right hindlimb were transiently paralyzed by injection of Botulinum toxin A (Botox). We observed that bone loss in this model was focal and prolific.

Methods

In vivo model

All mice (female C57BL/6) were 16 weeks of age at the start of the experiment. On day 0, anesthesia was induced by inhaled isoflurane (2%). While anesthetized, Botox (19.2 μ l of 2.0 unit/100 g, 2.5 unit/100 μ l) or saline (19.2 μ l) was injected (IM) into: (1) the right quadriceps 4 mm proximal to the patellar tendon (targeting the rectus femoris, vastus lateralis, vastus intermedius, and the vastus medialis) and (2) the posterior compartment of the right calf (targeting the gastrocnemius, plantaris, and the soleus). The Botox dose was based upon previous reports in the literature [4,35,38]. The animals were monitored continuously for the 5 min necessary for full recovery from anesthesia and intermittently during the subsequent 6 h, to ensure effective single hindlimb paralysis without side effects. The behavioral response of each mouse was quantified on days 1, 3, and 7 and weekly thereafter using whole body weight measurement and assessment of gait disability. Using a multi-observer inventory, gait disability was assessed as follows: (1) hindlimb abduction during tail suspension, (2) toe extension during sitting, (3) use of right leg during level walking, (4) use of right leg during two legged stance, and (5) use of right leg during climbing. With a range of 0–2 points for each of the 5 observations, the total possible score for each observation ranged from “0” (completely disabled) to “10” (normal). Animals were observed in random order and the same three observers assessed disability while blinded to the treatment groups. The within observer coefficient of variability for the inventory was 13.1%, with the inter-rater agreement of the inventory demonstrating a Cronbach's alpha of 0.997 for all observations [2]. During the study, mice were group housed with free ambulation and standard food and water ad-libitum. All mice received calcein injections on days 13 and 19 (IP, 10 mg/kg). Sacrifice occurred 21 days following Botox injection. All procedures were approved by the University of Washington IACUC.

Whole tissue assays

For each mouse, experimental and contralateral hindlimb muscle wet mass was assessed immediately following sacrifice for the quadriceps (vastus lateralis, medialis, intermedius, and rectus femoris) and calf muscle groups (gastrocnemius, soleus, plantaris). Also, femoral and tibial lengths were assessed by calipers (mm). For tibial length, 5 mice per group were available for assessment due to a tissue processing error.

Micro-CT imaging

A Scanco μ CT 20 high resolution micro-CT scanner was used to obtain 10.5 μ m voxel resolution images spanning the distal femur, the proximal tibia, and tibia mid-shaft of experimental and contralateral hindlimbs. Specific regions of analysis included 0.4 mm thick section spanning the distal femoral epiphysis, a 0.8 mm thick section spanning the proximal tibia metaphysis, and a 1 mm thick section of mid-diaphyseal cortical bone centered at 1.7 mm proximal to the tibia–fibula junction, similar to previous studies [18]. The epiphysis and metaphysis regions were identified by the onset and completion of the growth plate as determined via assessment of serial slices and recorded as the growth plate height (mm). Image noise was reduced with a low pass filter and bone identified using a fixed threshold. User guided contour subroutines were used to isolate trabecular bone from the cortical shell at the trabecular sites, while automated contour subroutines were used to define bone at the cortical sites. Bone histomorphometric analyses that utilize direct distance transformation methods to determine microarchitecture morphology were implemented at the trabecular sites [15,42]. Trabecular parameters included tissue volume (mm^3), bone volume (mm^3), bone volume/tissue volume (BV/TV; %), trabecular number (#/mm), thickness (mm), and spacing (mm). Additionally, the cortical shell surrounding the trabecular sites was separately analyzed to determine cortical shell periosteal volume (mm^3), with the cortical shell bone volume (mm^3) determined by subtracting the tissue volume (in this case serving to define the endocortical surface of the cortical shell) from the cortical shell periosteal volume. At the cortical sites, periosteal volume (mm^3), cortical volume (mm^3), endocortical volume (mm^3), and average cortical thickness (based upon the ratio of bone volume to bone surface, mm) were each quantified. Reproducibility assessment performed on three randomly selected bones in triplicate indicated that the coefficient of variation for tissue volume, bone volume and BV/TV, trabecular number, thickness, and spacing were 1.0, 5.2, 4.2, 5.1, 1.3, and 3.7%, respectively. For assessment of periosteal, cortical, and endocortical volume and cortical thickness, coefficients of variations were determined to be 0.1, 0.1, 0.1, and 0%, respectively. All imaging and analyses were conducted with the identity of the specimens blinded.

Dynamic histomorphometry

Following micro-CT imaging, sections were taken from mid-diaphysis of all tibia and ground to an 80 μ m thickness. Sections were imaged using a fluorescent microscope (40 $\times$), with identity of the sections blinded. On both the endocortical and periosteal surfaces, single-labeled surface (sLS), double-labeled surface (dLS), and interlabel thickness (Ir.L.Th) were measured. From these data, mineralizing surface ($\text{MS} = (\text{dLS} + \text{sLS}/2)/\text{BS} * 100$, with BS = bone surface), mineral apposition rate ($\text{MAR} = \text{Ir.L.Th}/\text{Ir.L.t}$, with Ir.L.t. = interlabel time period), and surface referent bone formation rate ($\text{BFR} = \text{MAR} * \text{MS}/\text{BS}$) were calculated [28].

Statistical analysis

Paired dependent *t* tests were used to assess potential differences between left and right hindlimbs of the saline mice, with no differences noted. Treatment effects were therefore determined using independent *t* tests to compare the injected (right) hindlimbs across the saline and Botox groups. Percent differences are reported as the difference between Botox and saline control relative to the control such that negative values reflect treatment related bone loss.

Repeated measures ANOVA was used to compare body mass (2×6 ; group $\times$ time) and observation scores (2×5) between groups over time. Data are reported as means and standard errors with the level of significance noted.

Results

At the onset of the study, the mice weighed an average of 22.2 ± 1.1 g. The Botox mice demonstrated a transient weight loss after the injection that peaked at 14 days (-5.5% vs. 0 day, $P < 0.01$), but returned to initial levels by 21 days (Fig. 1). Saline injected animals experienced a gradual increase in body mass through 21 days ($+7.0\%$ vs. 0 d; $P < 0.05$). Lameness, as assessed by locomotion inventory, ensued within 1 day of the Botox injections and was maximal by 3 days (Fig. 2). Gait dysfunction was demonstrated via an inability to spread toes while seated, holding or dragging the foot while walking, and minimized weight bearing while standing and climbing. Although function was still reduced compared to saline mice, gait disability in the Botox mice was significantly improved by 14 days. At this time, the Botox mice were able to flatten their toes while sitting, lift and place the right foot while walking, and partially bear body weight while standing and climbing.

Botox treatment significantly diminished right hindlimb muscle mass in both the quadriceps (102.9 ± 31.2 vs. 195.3 ± 13.9 mg, -47.3% , $P < 0.001$) and calf (58.4 ± 13.3 vs. 145.0 ± 3.9 mg, -59.7% , $P < 0.001$) compared to saline mice. Muscle mass in the contralateral hindlimb of the Botox treated mice was also significantly diminished compared to saline mice, but to a lesser extent (quadriceps: 166.0 ± 5.9 vs. 199.9 ± 2.7 mg, -17.0% ; calf: 120.8 ± 4.2 vs. 145.0 ± 1.6 mg, -16.7% , both $P < 0.001$). No differences in femur length, distal femur growth plate height, tibial length, or proximal tibia growth plate height were observed due to Botox treatment (Table 1).

Bone in the trabecular regions was substantially degraded by the Botox induced muscle paralysis (Table 2, Fig. 3). At the distal femoral epiphysis, tissue volume was unchanged by Botox treatment (-1.3%). The substantial BV/TV reduction (-43.2% , $P < 0.001$) was therefore achieved via a -43.8% decrease in bone volume ($P < 0.001$). BV/TV differences were not significant across contralateral femurs (7.3% , $P = 0.55$). Trabecular thickness in the Botox injected hindlimb was significantly diminished compared to saline mice (-25.2% , $P < 0.001$). The contralateral femur of the Botox treated mice demonstrated a consistent, but smaller, reduction in trabecular thickness compared to saline mice (-6.3% , $P = 0.01$). Neither trabecular number nor trabecular spacing was altered in the Botox treated hindlimbs compared to saline mice. The cortical shell bone volume surrounding the femoral epiphysis was significantly decreased in the Botox treated mice compared to saline mice (-12.1% , $P = 0.02$). Cortical shell alterations between the contralateral distal femurs were not statistically different.

Botox induced bone degradation was also substantial at the proximal tibia metaphysis (Table 2, Fig. 3). BV/TV was reduced -54.3% ($P < 0.001$) in Botox treated hindlimbs compared to saline mice. This loss was achieved by a combination of decreased bone volume (-46.0% , $P < 0.01$) and increased tissue volume (17.1% , $P < 0.001$). BV/TV, bone volume, and tissue volume were unchanged between contralateral tibiae. As was observed at the femoral epiphysis, Botox treatment significantly diminished trabecular thickness (-24.8% , $P < 0.001$), but did not significantly alter trabecular number or spacing. None of these parameters were significantly altered in the contralateral limbs. Cortical shell bone volume was significantly diminished when Botox and saline treated limbs were compared (-19.8% , $P < 0.001$). This loss of tissue arose primarily due to an expanded tissue volume (i.e., expanded endocortical surface of the cortical shell), as the cortical shell periosteal volume of the tibia metaphysis was not effected by Botox treatment. A similar pattern was observed in the contralateral proximal

tibias, as the cortical shell periosteal volume did not differ statistically, but the cortical shell bone volume was significantly diminished (-11.0% , $P < 0.01$).

At the tibia mid-diaphysis, low levels of osteoblast activity were observed in all mice resulting in large percentage differences across groups. Botox treatment reduced periosteal MS and BFR (-40.9% and -33.5% , respectively), but neither decrease was statistically significant. When non-injected contralateral limbs were compared, Botox mice demonstrated reduced periosteal MS (-59.5% , $P = 0.04$), MAR (-64.3% , $P = 0.05$), and BFR (-82.0% , $P = 0.08$) compared to saline mice. Endocortical MS, MAR, and BFR were all diminished by Botox treatment (-41.6% , -15.3% , -35.4% , respectively), but only MS reached statistical significance (Table 3). Endocortical MS was unchanged in contralateral limbs, and while MAR and BFR increased compared to saline contralateral limbs, neither approached statistical significance.

The volume of cortical bone at the tibia mid-diaphysis was significantly decreased by Botox treatment (-14.6% , $P < 0.001$, Table 3, Fig. 3). The loss of cortical bone was achieved primarily by expansion of the endocortical envelope ($+13.1\%$, $P < 0.01$), as periosteal volume was minimally altered (-2.9%). Concomitantly, the thickness of the cortex was significantly diminished at 21 days (-16.2% , $P < 0.001$). When contralateral non-injected tibia was contrasted, no statistically significant cortical bone alterations were observed with the exception of cortical thickness, which was reduced in Botox mice (-4.3% , $P < 0.01$).

Discussion

We observed rapid muscle and bone degradation in response to acute muscle paralysis induced by hindlimb Botox injections despite significant re-ambulation within the 21 day experiment. The muscle atrophy observed in the Botox mice exceeded that induced by non-surgical models of disuse such as hindlimb suspension [32], but was similar to that observed in more invasive tenotomy and neurectomy models [16,45]. As well, bone loss in the Botox mice was more severe than that observed in C57 mice challenged by hindlimb suspension [18]. Taken as a whole, the micro-CT and cortical histomorphometry data provide suggestive evidence that the trabecular and cortical bone loss observed in the model arose primarily via substantial bone resorption, rather than diminished skeletal growth. If confirmed at the cellular level in subsequent studies at earlier time points, this pattern of bone loss would mimic that observed in humans exposed to acute disuse [5] and would contrast with current rodent models of unloading in which bone loss arises via equal contributions from decreased formation and increased resorption [17,44].

Botox blocks the release of acetylcholine into the neuromuscular junction and thereby temporarily inhibits muscle contraction by the nervous system [13]. Upon intramuscular injection, Botox (serotype A) action is primarily local, with minimal diffusion to other tissues [41]. Acutely, muscle electrical activity is reduced within hours of IM injection and site specific muscle contractions are nearly completely inhibited by 18 h [6]. The duration of paralysis is dependent upon the dose of Botox. With lower doses, original motor neurons eventually regain exocytic capacities, while higher dose requires sprouting of new nerve terminals to re-establish neuromuscular connections [43]. Previous Botox investigations have reported doses ranging from 7 to 60 units per 100 g in humans with the higher doses used to reduce limb spasticity [35]. Botox studies in rodents have reported injection doses ranging from as low as 0.4 units per 100 g IM to 18 units per 100 g in the urinary tract [4,38]. Our dose of 2 units per 100 g was therefore at the low end of doses utilized in previous studies.

To our knowledge, the only previous study in which bone loss was assessed in response to Botox treatment was performed in 5 month old Wistar rats [4]. Following injection into the quadriceps, bone mineral content (BMC), and texture analysis of radiographs in the femur and

tibia were assessed after 4 weeks. Trabecular patterns of the distal femur and proximal tibia were altered, yet BMC was only significantly different (−17%) in the proximal tibia region when injected legs were compared with contralateral legs. A number of methodological differences may account for the more subtle alterations observed in the previous study, including injection only in the quadriceps (in our hands, this results in only moderate hindlimb unloading, data not shown) and lower resolution assays to detect bone alterations.

Although bone degradation was pronounced in the Botox treated limbs, analysis of contralateral limb data does suggest the presence of systemic effects in the model. It is likely that the systemic effects arose via the combined influence of the transient weight loss and reduced activity levels observed in the Botox treated mice. The observed weight loss was consistent both with that observed in previous animal studies examining Botox as well as alternate rodent models of disuse [4,25]. The loss of hindlimb muscle mass accounted for 10% of the difference in final body weight between the Botox and saline mice in our study. Botox treated mice also demonstrated diminished general activity levels during the first few days following treatment (observationally, activity of the Botox mice followed their level of lameness, Fig. 2). Although we did not specifically quantify caloric intake or activity levels in this study, we would anticipate that differences between treatment limbs and contralateral limbs would be similar in magnitude if systemic influences were primarily driving the observed tissue adaptation. Instead, we found that 14 of 22 tissue level parameters were significantly decreased in Botox treated limbs compared to saline treated limbs but only a subset of tissue parameters (5 of 22) demonstrated statistically significant decreases in the contralateral limbs of Botox treated mice compared to saline mice (quadriceps and calf muscle mass, trabecular thickness at the distal femur, cortical shell bone volume at the proximal tibia, and cortical thickness at the tibia mid-shaft). The average percentage decrease between contralateral limbs for this subset of parameters was 36% of that observed between treated limbs (range: 25 to 56%). These data suggest that the local bone loss induced by muscle paralysis exceeded that induced by systemic influences. In the future, it may be possible to reduce systemic influences in the model by achieving muscle paralysis via a reduced dose of Botox. If so, it may be possible to simultaneously explore both the local effects of muscle paralysis and systemic effects of reduced activity in the model.

With regard to the cortical bone dynamic histomorphometry, the large percentage alterations observed in MS, MAR, and BFR at the tibia mid-diaphysis arose primarily due to fairly low levels of osteoblast activity (e.g., only 3 of 10 saline mice demonstrated >5% periosteal double label averaged across both left and right tibia). The magnitude of MS, MAR, and BFR observed on the endocortical and periosteal surfaces of the saline mice in this study was consistent with values reported for like aged C57 mice in a previous study by our group and substantially lower than that observed in younger C57 mice [37,40]. If Botox directly affected periosteal lining cells and osteoblasts, this influence would be most dramatic on the periosteum near the injection sites. However, we found that periosteal MS, MAR, and BFR in the contralateral tibia of the Botox treated mice were less than that measured in the Botox treated tibia. In our view, this result was quite counterintuitive. If this observation was supported in future studies, it may suggest that bone loss induced by muscle paralysis and bone loss associated with diminished activity may be driven by distinct signaling pathways. Endocortical osteoblast function was diminished in the Botox treated limbs, as would be anticipated given the substantial osteoclastic activity implied by the morphologic changes observed in the model. A clear caveat of our histomorphometric analysis is that our assessment was confined to a single site at a single time interval near the end of the 3 week experiment. However, in the context of the tissue level data indicating that Botox treatment did not induce alterations in cortical shell periosteal volume at the trabecular sites, periosteal volume at the tibia mid-diaphysis, bone lengths, or growth plate heights, the potential that bone loss in the model arose via diminished skeletal growth is greatly

reduced. However, future studies will be required to refine this observation, particularly immediately following Botox injection and in mice demonstrating more robust bone formation.

The pathway by which acute muscle paralysis resulted in bone degradation in the Botox model remains to be elucidated. Tissue deformations in bone, which underlie the ability of loading to maintain bone homeostasis, are induced by a combination of ground reaction forces and muscle contraction. During hindlimb suspension or casting, or following neurectomy or spinal cord injury, animals are unable to utilize their hindlimbs for weight bearing. Current models of disuse therefore entail either complete removal of all ground reaction and muscle forces (spinal cord injury) or removal of ground reaction forces while muscle contraction remains active (tail suspension and casting). Interestingly, an initial study in rabbits suggests that peak ground reaction forces are only subtly altered when the quadriceps muscle function was inhibited by Botox injection [14]. Alternatively, the intimate physical proximity of muscle and bone suggests that biochemical factors released from contracting muscle (e.g., IL-6, TGF- β , TNF- α , VEGF, glutamate, calcitonin gene-related peptide (CGRP), or substance P) may also serve to enable bone homeostasis. It is likely that Botox reduces the full spectrum of normal muscle contractions (both small magnitude, high frequency (twitch), and large magnitude contractions) and thus diminishes the release of biochemical factors (or their transport) into the local muscle/bone milieu. As a result, we believe this model has substantial potential for mechanistic exploration of muscle/bone interactions. Specifically, techniques such as electrical muscle stimulation and/or direct deformation of bone [12] could be superimposed upon muscle paralysis to isolate the specific mechanical stimuli required to maintain bone homeostasis. Alternately, use of transgenic mice will enable unique delineation between mechanical and biochemical pathways responsible for controlling bone homeostasis and the attainment of peak bone mass.

In addition, these data also raise a health care issue of potential clinical significance. The use of neuromuscular inhibitors has rapidly progressed from solely cosmetic indications to broad treatment for musculo-skeletal pain or muscle spasticity [8,26]. While these interventions hold potential to bring substantial relief to patients, it is worrisome that bone loss associated with elective muscle paralysis has not been assessed in any clinical trial to date. Given that neuromuscular inhibitors are being increasingly utilized adjacent to locations such as the spine and that the rate of bone degradation observed in our study substantially exceeds that observed during menopause, it is reasonable to suggest that repeated acute muscle paralysis may predispose patients toward osteoporosis. As such, our data highlight the critical need to prospectively monitor bone loss in patients using Botox and other neuromuscular inhibitors especially near the spine, proximal femur, or mandible.

In conclusion, we have developed a murine model of unilateral transient muscle paralysis that demonstrates rapid and prolific degradation of both skeletal muscle and bone, confirming the decisive role of muscle contraction in maintaining bone mass. Indirectly, we observed that bone loss in the model appeared to be primarily driven via resorptive degradation, similar to human conditions of disuse. Further, while Botox treated mice greatly reduced the use of their right hindlimb within 3 days, they resumed partial weight bearing within 7 days, similar to what is experienced following knee or hip surgery. Given that the observed bone loss was indirectly induced by transiently inhibiting muscle activity, we believe that this model holds unique potential to specifically explore the role of muscle function in maintaining bone mass.

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Fig. 1.

Botox and saline group body mass variations. Mean body mass ($\pm$ SE) in the saline group was significantly elevated following day 0 ($*P < 0.05$). Body mass transiently decreased by 3 days in Botox treated mice, but returned to initial levels by 21 days ($\dagger P < 0.05$ vs. day 0). As a result, Botox mice weighed significantly less than saline mice from 1 day through 21 days ($\dagger P < 0.05$).

Fig. 2.

Gait function was diminished by Botox treatment. All Botox mice demonstrated significantly reduced function compared to saline mice ($*P < 0.01$). The observed disability due to Botox treatment was maximal by 3 days and was significantly improved by 14 and 21 days ($†P < 0.05$ vs. 1, 3, and 7 days).

Fig. 3. Micro-CT images (3-D) of the right distal femoral epiphysis, right proximal tibia metaphysis, and right tibia mid-diaphysis from a representative saline injected (top) and Botox injected mouse (bottom). Across groups, trabecular BV/TV (%) was reduced -43.2% and -54.3% at the distal femur and proximal tibia, respectively, and -14.6% at the tibia mid-diaphysis (all $P < 0.001$). The cortical shell periosteal volume at the distal femur and proximal tibia and the periosteal volume of the tibia mid-diaphysis did not differ between saline and Botox groups.

Table 1Bone length and growth plate height (mean $\pm$ SE)

		Saline	Botox	% difference	P value
Femur length (mm)	Injected	15.8 $\pm$ 0.1	15.9 $\pm$ 0.1	+0.6%	0.46
	Contralateral	15.8 $\pm$ 0.1	15.9 $\pm$ 0.1	+0.6%	0.32
Femur growth plate height (mm)	Injected	1.26 $\pm$ 0.02	1.25 $\pm$ 0.02	-0.8%	0.76
	Contralateral	1.23 $\pm$ 0.02	1.27 $\pm$ 0.02	+2.9%	0.27
Tibia length * (mm)	Injected	18.1 $\pm$ 0.1	18.1 $\pm$ 0.2	0.0%	1.00
	Contralateral	18.1 $\pm$ 0.1	18.0 $\pm$ 0.1	-0.7%	0.41
Tibia growth plate height (mm)	Injected	0.72 $\pm$ 0.01	0.70 $\pm$ 0.02	-2.2%	0.52
	Contralateral	0.74 $\pm$ 0.01	0.71 $\pm$ 0.01	-4.6%	0.06

* Independent *t* test Botox vs. saline injected hindlimbs compared (all parameters based upon *n* = 10 per group, except tibia length, *n* = 5 per group).

Table 2

Trabecular bone micro-CT data (mean $\pm$ SE)

		Saline	Botox	% difference	P value
<i>Femur distal epiphysis</i>					
Tissue volume (mm ³)	Injected	0.981 $\pm$ 0.074	0.969 $\pm$ 0.086	-1.3	0.74
	Contralateral	0.950 $\pm$ 0.026	0.930 $\pm$ 0.039	-2.1	0.67
Bone volume (mm ³)	Injected	0.262 $\pm$ 0.026	0.147 $\pm$ 0.064	-43.8	<0.001 ^a
	Contralateral	0.259 $\pm$ 0.007	0.244 $\pm$ 0.014	-5.9	0.35
Trabecular fraction (BV/TV, %)	Injected	26.65 $\pm$ 1.75	15.15 $\pm$ 6.69	-43.2	<0.001 ^a
	Contralateral	27.29 $\pm$ 0.51	29.28 $\pm$ 3.22	+7.3	0.55
Trabecular number (#/mm)	Injected	6.656 $\pm$ 0.2329	6.523 $\pm$ 0.401	-2.0	0.40
	Contralateral	6.647 $\pm$ 0.0635	6.767 $\pm$ 0.101	+1.8	0.33
Trabecular thickness (mm)	Injected	0.061 $\pm$ 0.0015	0.046 $\pm$ 0.009	-25.2	<0.001 ^a
	Contralateral	0.062 $\pm$ 0.0009	0.058 $\pm$ 0.001	-6.3	<0.01 ^a
Trabecular spacing (mm)	Injected	0.163 $\pm$ 0.0086	0.167 $\pm$ 0.013	+2.8	0.37
	Contralateral	0.159 $\pm$ 0.0019	0.156 $\pm$ 0.003	-1.6	0.48
Cortical shell periosteal volume (mm ³)	Injected	1.568 $\pm$ 0.030	1.485 $\pm$ 0.045	-5.3	0.14
	Contralateral	1.571 $\pm$ 0.027	1.524 $\pm$ 0.031	-3.0	0.26
Cortical shell bone volume (mm ³)	Injected	0.587 $\pm$ 0.011	0.516 $\pm$ 0.029	-12.1	0.02 ^a
	Contralateral	0.621 $\pm$ 0.020	0.594 $\pm$ 0.047	-4.3	0.64
<i>Tibia proximal metaphysis</i>					
Tissue volume (mm ³)	Injected	1.737 $\pm$ 0.123	2.034 $\pm$ 0.101	+17.1	<0.001 ^a
	Contralateral	1.819 $\pm$ 0.037	1.854 $\pm$ 0.063	+1.9	0.64
Bone volume (mm ³)	Injected	0.111 $\pm$ 0.028	0.060 $\pm$ 0.046	-46.0	0.01 ^a
	Contralateral	0.115 $\pm$ 0.007	0.125 $\pm$ 0.017	+9.3	0.56
Trabecular fraction (BV/TV, %)	Injected	6.32 $\pm$ 1.26	2.89 $\pm$ 0.68	-54.3	<0.001 ^a
	Contralateral	6.34 $\pm$ 0.41	6.62 $\pm$ 2.21	+4.3	0.74
Trabecular number (#/mm)	Injected	3.364 $\pm$ 0.231	3.207 $\pm$ 0.334	+5.0	0.24
	Contralateral	3.507 $\pm$ 0.069	3.519 $\pm$ 0.104	+0.4	0.92
Trabecular thickness (mm)	Injected	0.049 $\pm$ 0.045	0.037 $\pm$ 0.044	-24.8	<0.001 ^a
	Contralateral	0.050 $\pm$ 0.001	0.047 $\pm$ 0.001	-5.2	0.11
Trabecular spacing (mm)	Injected	0.294 $\pm$ 0.025	0.314 $\pm$ 0.036	+6.9	0.16
	Contralateral	0.283 $\pm$ 0.006	0.285 $\pm$ 0.010	+0.8	0.84
Cortical shell periosteal volume (mm ³)	Injected	2.985 $\pm$ 0.050	3.034 $\pm$ 0.028	1.7	0.37
	Contralateral	3.075 $\pm$ 0.045	2.972 $\pm$ 0.073	-3.4	0.26
Cortical shell bone volume (mm ³)	Injected	1.248 $\pm$ 0.037	1.001 $\pm$ 0.015	-19.8	<0.001 ^a
	Contralateral	1.256 $\pm$ 0.030	1.118 $\pm$ 0.036	-11.0	0.01 ^a

^aIndependent *t* test Botox vs. saline injected hind limbs compared.

Table 3

Cortical bone histomorphometry and micro-CT data (mean $\pm$ SE)

		Saline	Botox	% difference	P value
p.MS (%)	Injected	10.5 $\pm$ 2.6	6.3 $\pm$ 1.5	-40.0	0.18
	Contralateral	11.6 $\pm$ 2.8	4.7 $\pm$ 1.4	-59.5	0.04 ^a
p.MAR ($\mu\text{m}/\text{day}$)	Injected	0.32 $\pm$ 0.10	0.32 $\pm$ 0.14	-1.9	0.97
	Contralateral	0.42 $\pm$ 0.10	0.15 $\pm$ 0.08	-64.3	0.05 ^a
p.BFR ($\mu\text{m}^3/\mu\text{m}^2/\text{year}$)	Injected	15.8 $\pm$ 7.1	10.5 $\pm$ 5.1	-33.5	0.55
	Contralateral	22.1 $\pm$ 8.3	4.8 $\pm$ 3.8	-82.0	0.08
e.MS (%)	Injected	39.9 $\pm$ 5.3	23.3 $\pm$ 5.8	-41.6	0.05 ^a
	Contralateral	35.5 $\pm$ 4.0	36.4 $\pm$ 5.6	+2.5	0.96
e.MAR (μ/day)	Injected	0.72 $\pm$ 0.05	0.61 $\pm$ 0.17	-15.3	0.53
	Contralateral	0.58 $\pm$ 0.12	0.78 $\pm$ 0.14	+34.5	0.31
e.BFR ($\mu\text{m}^3/\mu\text{m}^2/\text{year}$)	Injected	111.2 $\pm$ 19.0	80.1 $\pm$ 26.0	-35.4	0.35
	Contralateral	81.5 $\pm$ 21.3	122.1 $\pm$ 27.7	+49.8	0.28
Periosteal volume (mm^3)	Injected	0.993 $\pm$ 0.019	0.964 $\pm$ 0.018	-2.9	0.29
	Contralateral	1.025 $\pm$ 0.011	1.010 $\pm$ 0.022	-1.4	0.55
Cortical volume (mm^3)	Injected	0.602 $\pm$ 0.008	0.514 $\pm$ 0.012	-14.6	<0.001 ^a
	Contralateral	0.612 $\pm$ 0.007	0.589 $\pm$ 0.013	-3.7	0.13
Endocortical volume (mm^3)	Injected	0.391 $\pm$ 0.015	0.450 $\pm$ 0.009	+13.1	<0.01 ^a
	Contralateral	0.413 $\pm$ 0.022	0.421 $\pm$ 0.010	-2.3	0.51
Cortical thickness (mm)	Injected	0.208 $\pm$ 0.003	0.174 $\pm$ 0.004	-16.2	<0.001 ^a
	Contralateral	0.211 $\pm$ 0.002	0.202 $\pm$ 0.002	-4.3	<0.01 ^a

^aIndependent *t* test Botox vs. saline injected hind limbs compared.