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ORIGINAL RESEARCH

Combined Effects of Botulinum Toxin Injection and Hind Limb Unloading on Bone and Muscle

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Abstract Bone receives mechanical stimulation from two primary sources, muscle contractions and external gravitational loading; but the relative contribution of each source to skeletal health is not fully understood. Understanding the most effective loading for maintaining bone health has important clinical implications for prescribing physical activity for the treatment or prevention of osteoporosis. Therefore, we investigated the relative effects of muscle paralysis and reduced gravitational loading on changes in muscle mass, bone mineral density, and microarchitecture. Adult female C57Bl/6J mice ($n = 10$/group) underwent one of the following: unilateral botulinum toxin (BTX) injection of the hind limb, hind limb unloading (HLU), both unilateral BTX injection and HLU, or no intervention. BTX and HLU each led to significant muscle and bone loss. The effect of BTX was diminished when combined with HLU, though generally the leg that received the combined intervention (HLU+BTX) had the most detrimental changes in bone and muscle. We found an indirect effect of BTX affecting the uninjected (contralateral) leg that led to significant decreases in bone mineral density and deficits in muscle mass and bone architecture relative to the untreated controls; the magnitude of this indirect BTX effect was comparable to the direct effect of BTX treatment and HLU. Thus, while it was difficult to definitively conclude whether muscle force or external gravitational loading contributes more to bone maintenance, it appears that BTX-induced muscle paralysis is more detrimental to muscle and bone than HLU.

Keywords Disuse · Botulinum toxin · Mechanical loading · Tail suspension · Muscle–bone interaction · Hind limb unloading · Paralysis

Introduction

Mechanical forces on bone, which are critical to skeletal health, derive from two primary sources: external gravitational loading via ground reaction forces and internal loading via muscle contractions. The relative contribution of muscle forces and external loading to skeletal health is still debated [1–3], calling into question the theory advanced by Frost [4] that muscle forces dominate bone adaptation since they exert the largest forces on the skeleton. Understanding the most effective means of stimulating bone formation or maintaining bone health via mechanical loading has important clinical implications for prescribing physical activity for the treatment or prevention of osteoporosis.

Many different experimental unloading methods have been used to manipulate the mechanical environment of

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bone in rodents, to improve our understanding of how bone responds to mechanical forces. Rodent disuse models can roughly be divided into two categories: (1) those that remove or reduce external ground reaction forces but spare muscle activation (e.g., hind limb unloading [HLU] [5], limb immobilization [6], cast immobilization [7], partial weight suspension [8]) and (2) those that eliminate muscle contractions but permit external forces (e.g., botulinum toxin [BTX] [9], neurectomy [10], tendon resection [11]). Although these two categories are useful for generally characterizing the disuse models, it is clear that the in vivo situation is more complex than implied by these two broad categories. Indeed, muscle and external forces are intricately linked in vivo; and thus, it is impracticable to manipulate one loading modality without affecting the other. For instance, muscle contractions are permitted in the HLU model, but muscle forces are theoretically reduced since they need not oppose the torque of the ground reaction forces. Analogously, in the BTX model, intramuscular injection of BTX in hind limbs elicits temporary muscle paralysis, which secondarily alters gait and impacts external forces such that peak ground reaction forces are reduced by 11 % 4 days after BTX injection [12]. These limitations notwithstanding, these models are valuable tools to study the relative musculoskeletal effects of the removal of ground reaction forces versus the removal of muscle forces.

In this regard, bone deterioration following muscle paralysis via BTX injection is purportedly more rapid and extreme than that seen with removal of ground reaction forces via HLU [9, 13]. In contrast with this assertion, Warden et al. [14] concluded that HLU has a greater skeletal effect than BTX injection based on a study combining HLU and BTX injection. However, Warden et al. did not include normally loaded or HLU control groups without BTX injection to be able to address the independent effects of muscle paralysis relative to HLU.

To address the gap in knowledge regarding the relative influence of external forces and muscle forces on skeletal health, we removed one or both sources of mechanical stimulus and studied the resulting bone and muscle changes in adult mice. BTX-A injection into the primary extensors of the left hind limb was used to eliminate internal muscle forces, whereas HLU was used to eliminate external ground reaction forces. An uninjected, normal, cage-dwelling group was also included as a control. We included a group receiving both interventions combined to evaluate if either mechanical stimulus acting alone, i.e., in the groups receiving a single intervention, limits bone loss relative to a condition of extreme disuse. We hypothesized that BTX-induced muscle paralysis would have a more detrimental effect on the skeleton than HLU and that the combination of paralysis and unloading would have a worse effect on muscle mass, bone mineral density (BMD), and bone microarchitecture than either intervention alone.

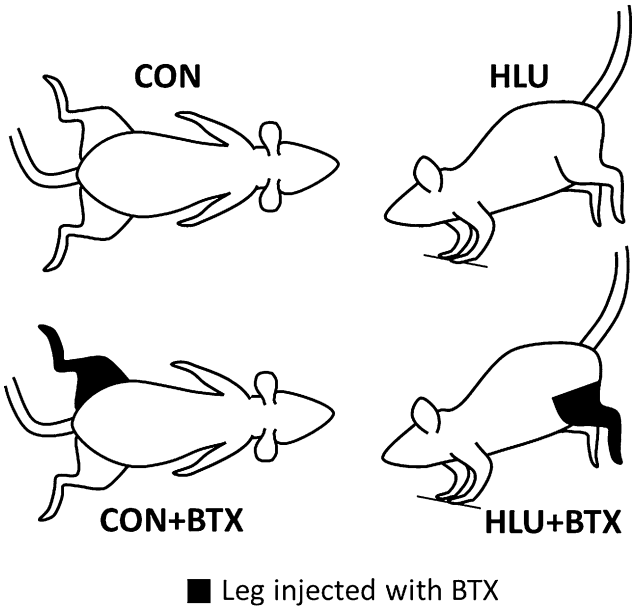

Fig. 1 Schematic of experimental design of four groups: *CON* untreated and cage-dwelling, *HLU* untreated and hind limb–unloaded, *CON+BTX*, injected with BTX in left leg and cage-dwelling; *HLU+BTX*, injected with BTX in left leg and hind limb–unloaded

Materials and Methods

Experimental Design

Eleven-week-old, female C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME) were assigned by body mass and total-body BMD (TBBMD) to one of two housing conditions ($n = 20$ each): (1) cage control group-housed in standard vivarium cages or (2) HLU. These groups were then further divided ($n = 10$ each) with half receiving injections of BTX in one leg (CON+BTX, HLU+BTX) and the other half receiving no injections (CON, HLU; Fig. 1). HLU was initiated on day 0, and BTX injections were performed 3 days prior so that the mice would have maximal paralysis at the start of the unloading period. All groups were provided with standard chow and water ad libitum. The diets of BTX-injected groups were supplemented with DietGel 76A and Hydrogel (ClearH2O, Portland, ME) on the cage bottom to aid in access to food and water. Body mass was monitored daily for the first week and three times weekly thereafter. On day 21, mice were euthanized via carbon dioxide inhalation. The protocol was approved by the Beth Israel Deaconess Medical Center institutional animal care and use committee.

Unilateral Hind Limb Muscle Paralysis by BTX Injection

Three days prior to the start of the experiment, under inhaled isoflurane anesthesia, the left leg of CON+BTX and HLU+BTX mice was injected with BTX-A (2.5 U/100 μL, BOTOX; Allergan, Irvine, CA) in the quadriceps muscle group and the triceps surae, or calf, muscle group (10 μL/muscle group). A total dose of 2 U/100 g was chosen to be consistent with past studies [9, 14, 15]. The contralateral leg served as an internal control and was not injected with saline since past studies showed no negative effects from saline injection [9, 16]. On day 1 of HLU (i.e., day 4 post–BTX injection), the HLU+BTX group had significant weight loss and low activity, so each mouse was given a subcutaneous injection of 0.6 mL lactated Ringer's solution for 2 days.

The degree of muscle paralysis was assessed in HLU+BTX and CON+BTX groups on days -2, 0, 4, 7, 11, 14, and 18 using digit abduction scoring (DAS) and a custom wire hang test. DAS was performed per the specifications of Aoki [17], in which mice are briefly suspended by the tail to elicit a startle response comprising hind limb extension and hind digit abduction. HLU animals, already tail-suspended, were raised further by their tail until startled. The DAS assay was scored on a scale of 0–4, where a score of 0 indicated normal digit abduction and a score of 4 indicated maximal reduction in digit abduction with a curved foot and all five digits touching. A custom wire hang test was also used to evaluate upper hind limb strength and complement the DAS in the event that the HLU+BTX mice were unable to be startled after growing accustomed to tail suspension. In this test, mice were individually placed on top of a wire cage insert, the insert was overturned, and the animal's use of its injected hind limb was scored on a scale of 0–3, where 0 indicated normal ability to grip wire and hang body weight from the injected hind limb and 3 indicated inability to flex the hip and/or extend the leg to touch the foot to the wire cage insert. An intermediate score of 2 indicated ability to bring the leg toward the wire cage insert but not accurately place the foot on wire, and with a score of 1 the mouse could place the foot on the wire but not grip and support its weight. Two observers independently performed the DAS and wire hang test assessments at each time point, and their scores were averaged.

Hind Limb Unloading

On day 0, mice in the HLU and HLU+BTX groups were hind limb–unloaded via tail suspension following the recommendations of Morey-Holton and Globus [18–20]. Briefly, under isoflurane anesthesia, the tail was taped to a freely rotating harness connected to a wheel that could move along the central axis of the cage. The harness was adjusted such that the mouse could not touch its hind paws to the floor or the walls of the cage, leading to complete removal of all ground reaction forces. However, muscle contraction remained active in the non–BTX-injected limbs.

BMD by Dual-Energy X-Ray Absorptiometry

BMD (grams per square centimeter) of the total body (exclusive of the head) and both hind limbs (from femoral neck to ankle) was assessed by peripheral dual-energy X-ray absorptiometry (DXA, PIXImus II; GE Lunar, Madison, WI), as described previously [20], at baseline (day -3 or -4) and death.

Ex Vivo Muscle Measurements

Immediately after euthanasia, the gastrocnemius and soleus muscles were dissected bilaterally and wet mass was measured (±0.01 mg). Left and right muscle masses in untreated mice were averaged together.

Bone Microarchitecture by Micro-computed Tomography

Tibiae and femora were collected bilaterally from the BTX-injected groups, while only right-sided bones from the CON and HLU groups were analyzed, as BMD and muscle data confirmed there were no bilateral differences in the untreated groups. The bones were dissected, cleaned of soft tissue, and fresh frozen at −20 °C. Cortical and trabecular bone microarchitecture of the tibia and femur were assessed according to published guidelines [21] using high-resolution micro-computed tomography (μCT40; Scanco Medical, Bassersdorf, Switzerland) with a 12-μm isotropic voxel size, as described previously [22]. Images were acquired at 70 kVp and 114 mA, with 200 ms integration time. Three volumes were analyzed: proximal tibia metaphysis (beginning 120 μm distal to the proximal growth plate, extending 1,200 μm distally), midshaft tibia (600 μm long beginning 2 mm proximal to the tibiofibular junction), and distal femur metaphysis (beginning 240 μm proximal to the distal growth plate, extending 1,800 μm proximally). Gaussian filtration was applied to the gray-scale images ($\sigma = 0.8$, support = 1). Trabecular and cortical bone were identified using automated algorithms and segmented using a global threshold of 276 and 708 mg HA/cm^3, respectively. Morphological analyses were performed on the binarized images using direct, 3D techniques that do not rely on any assumptions about the underlying structure [23–25]. Morphometric variables of

cancellous bone included bone volume fraction (Tb.BV/TV, percent), trabecular thickness (Tb.Th, millimeters), trabecular number (Tb.N, per millimeter), structure model index (SMI), and degree of anisotropy (DA). Cortical bone morphology measurements included average cortical thickness (Ct.Th, millimeters), total cross-sectional area (Tt.Ar, square millimeters), cortical bone area (Ct.Ar, square millimeters), cortical area fraction (Ct.Ar/Tt.Ar, percent), and polar moment of inertia (*J*, millimeters to the fourth power).

Statistical Analysis

We used paired *t*-tests within each group to determine whether body mass, BMD, and paralysis scores changed from baseline to final. Differences in paralysis scores between the HLU+BTX and CON+BTX groups were analyzed using unpaired *t*-tests at each day. Differences in body mass among groups on a given day were analyzed using a two-way analysis of variance (ANOVA) for days 0, 1, 3, 7, and 21.

We defined a "direct effect" of BTX as the difference in outcomes between the BTX-injected leg and the uninjected (contralateral) leg within a mouse. To test for a direct effect of BTX and the influence of unloading, we performed a two-way ANOVA on data from both legs of the CON+BTX and HLU+BTX groups, with *unloading* (HLU vs. cage control) as a between-subject factor and *BTX injection* (BTX-injected vs. uninjected) as a within-subject factor. We interpreted a significant *unloading* × *BTX injection* interaction term to indicate that the direct effect of BTX depended on loading status. A paired *t*-test between the right and left limbs of BTX-injected mice was used to test for the direct effect of BTX within the HLU+BTX and CON+BTX groups.

We defined an "indirect effect" of BTX as the difference in outcomes between the uninjected leg of the BTX-treated mice and their respective untreated control groups. To test for an indirect effect of BTX and the influence of unloading, we performed a two-way ANOVA using data from only the uninjected legs of the CON+BTX and HLU+BTX groups and the legs from the CON and HLU groups, with *unloading* (HLU vs. cage control) and *BTX treatment* (BTX-treated vs. untreated) as between-subject factors. A significant *unloading* × *BTX treatment* interaction term indicated that the indirect effect of BTX depended on loading status. An unpaired *t*-test between the CON and HLU groups was used to test for the simple effect of unloading.

To examine the contribution of weight loss to the indirect effects of BTX, we performed a linear regression between percent change in body mass and musculoskeletal outcomes from the CON, HLU, and the uninjected leg of the CON+BTX and HLU+BTX groups.

Differences were considered significant when $p < 0.05$.

Results

One HLU+BTX mouse died before study completion and was excluded from all analyses.

Body Mass

Body mass was similar in all groups at baseline. CON+BTX and HLU+BTX mice lost weight steadily after BTX injection, such that on unloading day 0 (3 days after BTX injection) both groups weighed 11 % less than the CON and HLU groups (Fig. 2). Following initiation of suspension, HLU+BTX mice experienced further weight loss, reaching a nadir of −22 % 2 days after suspension, before rebounding to equal the weight of the CON+BTX group at the completion of the study. A small deficit in body mass remained in both BTX-treated groups by the end of the study ($p < 0.001$, day -3 vs. day 21). HLU mice had transient weight loss up to −10 % on day 3, similar to the effect of suspension on the HLU+BTX group, but by day 21 returned to their baseline weight and were equal to the weight of the CON group, whose body mass did not change throughout the experiment.

Paralysis

Limb paralysis as assessed by DAS ensued within 1 day of BTX injection and was maximal after 3 days (i.e., at the start of HLU), with complete loss of digit abduction in almost all mice (CON+BTX 3.80 ± 0.42, HLU+BTX 3.94 ± 0.17; Fig. 3a). Recovery was gradual over the 3 weeks of the study, with initial improvements in CON+BTX outpacing the HLU+BTX group (day 4, $p < 0.01$). DAS observations in the HLU+BTX group were unreliable after day 11 because it was not possible to elicit a startle response in all mice.

Deficits in wire hang ability appeared more gradually than DAS, but maximal paralysis also occurred on day 0 (3 days after BTX injection), with a complete inability to reach the foot to the wire in almost all mice (CON+BTX 2.75 ± 0.42, HLU+BTX 2.83 ± 0.35; Fig. 3b). There was a rapid recovery between days 0 and 7 that slowed thereafter, and only small deficits remained by day 18.

No paralysis was observed in the contralateral limb of BTX-injected mice.

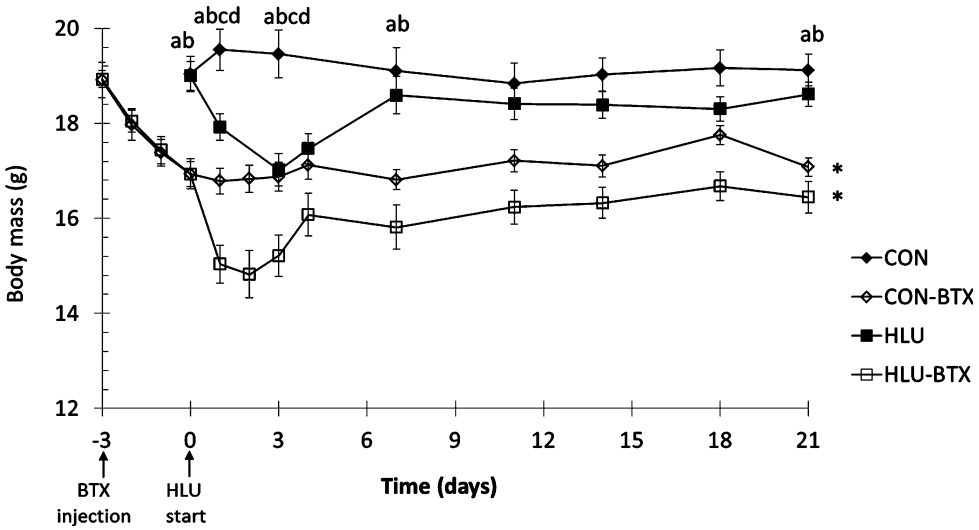

Fig. 2 Body mass changes following injection of BTX on day -3 and initiation of HLU on day 0 (mean ± SE). *Difference between day 21 and initial measurement. On a given day: *a* difference between CON and CON+BTX, *b* difference between HLU and HLU+BTX, *c* difference between CON and HLU, difference between CON+BTX and HLU+BTX

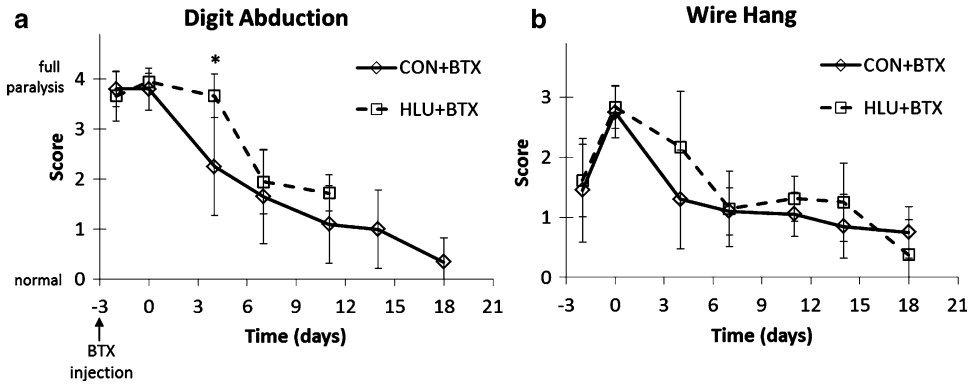

Fig. 3 Scores on **a** digit abduction and **b** wire hang assessments of muscle paralysis (mean ± SD). *Difference between groups by day

Muscle Mass

HLU alone led to large decreases in soleus mass (−41.6 %, HLU vs. CON, $p < 0.001$; Fig. 4a), with no further decrease when HLU was combined with BTX treatment ($p = 0.14$). In contrast, BTX injection in normally loaded mice led to a substantial decrease in soleus mass (−35 %, $p < 0.001$). Thus, the direct effect of BTX on soleus mass was greater in normally loaded mice than those exposed to HLU ($p_{\text{interaction}} < 0.001$).

Gastrocnemius mass (Fig. 4b) was less affected than soleus mass by HLU in untreated mice (−21.1 %, $p < 0.001$, HLU vs. CON). BTX did have a direct effect on the gastrocnemius when combined with HLU (−41.1 %, $p < 0.001$ right vs. left leg). In normally loaded mice, the BTX-induced decrease in gastrocnemius mass was even greater (−52.1 %, $p < 0.001$) than in HLU+BTX mice ($p_{\text{interaction}} < 0.0001$). Furthermore, there was no difference in gastrocnemius mass in injected limbs of CON+BTX and HLU+BTX mice, suggesting that BTX overwhelmed the effect of unloading. There was an indirect effect of BTX treatment on the gastrocnemius, such that gastrocnemius mass of the uninjected leg was 25–30 % lower ($p < 0.0001$) than in the respective untreated controls (Fig. 4b).

BMD

All groups were matched at baseline by total-body BMD, averaging 0.0461 ± 0.0013 g/cm^2. All further BMD data are from measurements of the hind limbs since the experimental disuse was intended to locally affect the hind limb.

In untreated mice, hind limb BMD increased in the CON group (+4.6 % vs. baseline, $p < 0.001$) and declined in the HLU group (−4.9 % vs. baseline, $p < 0.01$; Fig. 5) equally in both legs. The BMD decline in the injected leg of CON+BTX mice (−19.1 % vs. baseline, $p < 0.0001$) exceeded the decrease due to unloading alone ($p < 0.001$).

BTX injection had a profound direct effect on BMD as hind limb BMD declined approximately sixfold more in the injected leg of the HLU+BTX group (−30.2 % vs. baseline, $p < 0.0001$) than the HLU group. However, BMD also declined in the uninjected leg of the HLU+BTX group (−17.8 % vs. baseline, $p < 0.0001$), which exceeded the effect from HLU alone, confirming an indirect effect of the BTX injection. Further, the indirect negative effect of BTX

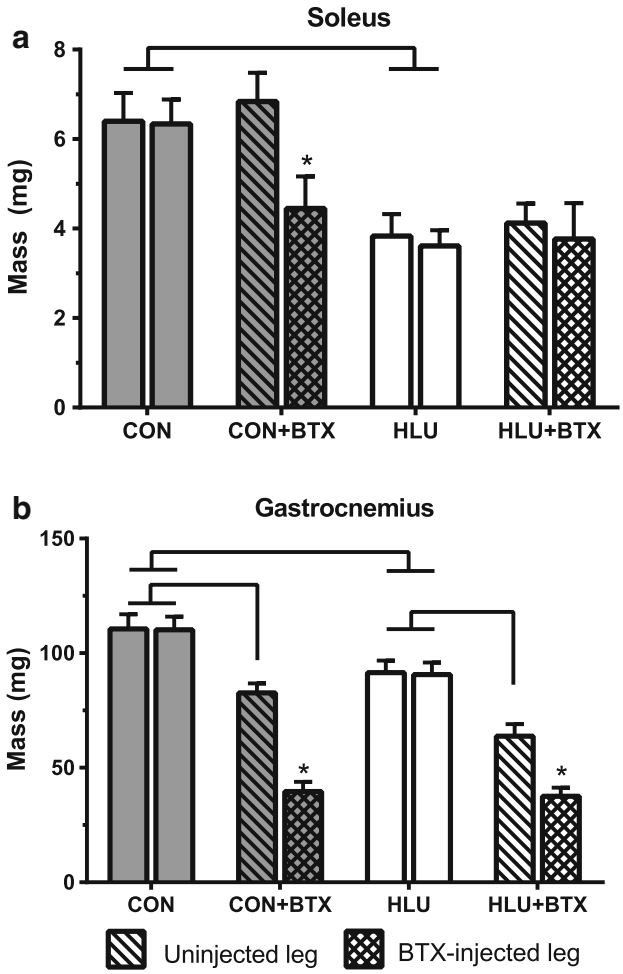

Fig. 4 Effect of BTX and HLU on **a** soleus and **b** gastrocnemius muscle mass (mean ± SD) *Brackets* indicate significant differences ($p < 0.05$) from respective control group. *$p < 0.05$, BTX-injected versus uninjected leg within loading group

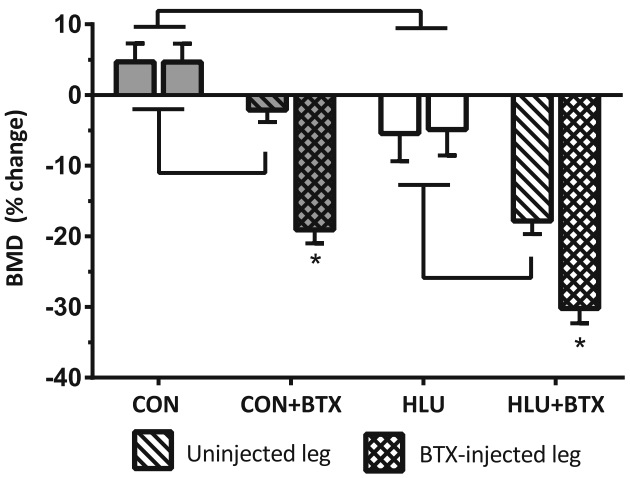

Fig. 5 Effect of BTX and HLU on hind limb BMD (percent change from *baseline*, mean ± SD). *Brackets* indicate significant differences ($p < 0.05$) from respective control group. *$p < 0.05$, BTX-injected versus uninjected leg within loading group

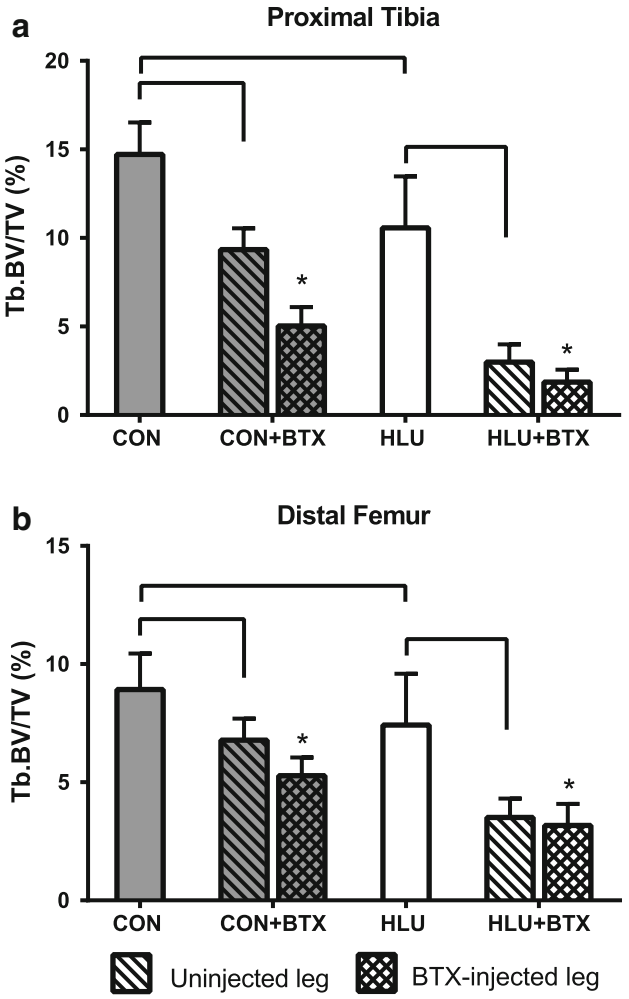

Fig. 6 Effect of BTX and HLU on trabecular bone volume fraction of the **a** proximal tibia and **b** distal femur (mean ± SD). *Brackets* indicate significant differences ($p < 0.05$) from respective control group. *$p < 0.05$, BTX-injected versus uninjected leg within loading group

was greater in the HLU+BTX group than in the CON+BTX group ($p_{\text{interaction}} < 0.01$). In both BTX-treated groups, there was a greater BMD decline in the injected versus the contralateral leg ($p < 0.0001$); however, the direct effect of BTX was greater for the CON+BTX group than the HLU+BTX group ($p_{\text{interaction}} < 0.001$).

Bone Microarchitecture

At the end of the 21-day unloading period, untreated HLU mice had significantly lower Tb.BV/TV (−28.2 %, Fig. 6a), Tb.Th (−11.0 %), and DA and higher SMI in the proximal tibia than the untreated CON group ($p < 0.01$ for all), with no differences in Tb.N (Table 1).

The direct effect of BTX injection on trabecular bone microarchitecture was twice that of unloading alone as Tb.BV/TV and Tb.Th of the CON+BTX injected leg were

Table 1 Trabecular bone microarchitecture of the proximal tibia (mean ± SD)

	CON		HLU	
	+BTX	−BTX	+BTX	−BTX
Tb.BV/TV [%]				
Inj	5.03 ± 1.0[abcd]		1.86 ± 0.7[d]	
Non	9.34 ± 1.2	14.72 ± 1.8[efg]	2.99 ± 0.7	10.57 ± 2.9
Tb.N [1/mm]				
Inj	3.79 ± 0.21[a]		2.85 ± 0.31	
Non	3.93 ± 0.30	4.24 ± 0.43[efg]	2.84 ± 0.40	3.86 ± 0.61
Tb.Th [mm]				
Inj	0.037 ± 0.003[abd]		0.030 ± 0.002[d]	
Non	0.049 ± 0.003	0.056 ± 0.003[ef]	0.040 ± 0.003	0.050 ± 0.005
SMI				
Inj	3.31 ± 0.20[acd]		3.68 ± 0.17[d]	
Non	3.04 ± 0.18	2.48 ± 0.24[efg]	3.95 ± 0.30	2.84 ± 0.30
DA				
Inj	1.64 ± 0.10[abd]		1.27 ± 0.06[d]	
Non	1.88 ± 0.11	2.00 ± 0.15[ef]	1.57 ± 0.11	1.82 ± 0.09

By ANOVA among both legs of BTX-treated groups, significant:

[a] Effect of *unloading*

[b] Effect of *BTX injection*

[c] *Unloading* × *BTX injection* interaction

[d] Paired *t*-test between uninjected and BTX-injected legs within group

By ANOVA among untreated controls and uninjected leg of BTX group, significant:

[e] Effect of *unloading*

[f] Effect of *BTX treatment*

[g] *Unloading* × *BTX treatment* interaction

+BTX BTX-treated groups, *−BTX* untreated controls, *Inj* injected leg, *Non* noninjected leg

−46 and −24 % lower, respectively, than values for the contralateral leg. In HLU+BTX mice, Tb.BV/TV (−42.1 %) and Tb.Th (−26.3 %) were also lower in the BTX-injected limb than the contralateral limb. The direct effect of BTX was significantly less in HLU+BTX mice than in normally loaded CON+BTX mice for Tb.BV/TV and SMI ($p_{\text{interaction}} < 0.0001$).

However, there was a substantial indirect effect of BTX leading to deficits in Tb.BV/TV, Tb.N, Tb.Th, SMI, and DA in the uninjected leg of both BTX-treated groups compared to their respective HLU and CON untreated control groups ($p < 0.02$ for all; Fig. 6a, Table 1). This indirect effect tended to be worse when combined with unloading ($p_{\text{interaction}} < 0.05$). For example, the deficit in Tb.BV/TV due to the indirect effects of BTX was −36.5 % for CON versus CON+BTX uninjected leg and −73.9 % for HLU versus HLU+BTX uninjected leg. The indirect effect of BTX in the HLU+BTX group exceeded the direct effects of BTX; thus, the differences in trabecular bone microarchitecture between the paired limbs of HLU+BTX were much smaller than the differences between the uninjected leg in the HLU+BTX group and the HLU group. Overall, the worst trabecular architecture was seen in the injected leg of the HLU+BTX group.

The differences in Tb.BV/TV at the distal femur were consistent with those at the proximal tibia (Fig. 6), with significantly lower trabecular bone resulting from unloading and indirect BTX treatment but with less of a direct BTX treatment effect than at the tibia.

At the tibial midshaft (Table 2), the HLU group had lower Ct.Th, Ct.Ar, Ct.Ar/Tt.Ar, and *J* than the CON group ($p < 0.05$) but did not differ in Tt.Ar. Greater deficits between the CON+BTX injected versus contralateral legs were observed for Ct.Th, Ct.Ar, and Ct.Ar/Tt.Ar ($p < 0.01$ for all) as a result of direct BTX treatment compared to unloading alone. As noted for prior outcomes, the effect of BTX was significantly less in the HLU+BTX group than the CON+BTX group for Ct.Ar and Ct.Th ($p_{\text{interaction}} < 0.02$).

There was a significant indirect effect of BTX on cortical bone morphology for every measure except Tt.Ar in both the CON+BTX and HLU+BTX groups as the uninjected legs

Table 2 Cortical bone microarchitecture of the tibial midshaft (mean ± SD)

		CON		HLU	
		+BTX	−BTX	+BTX	−BTX
Ct.Th [mm]	Inj	0.134 ± 0.012[abcd]		0.121 ± 0.012[d]	
	Non	0.163 ± 0.009	0.178 ± 0.005[ef]	0.136 ± 0.007	0.158 ± 0.011
Tt.Ar [mm^2]	Inj	0.845 ± 0.049[b]		0.859 ± 0.045[d]	
	Non	0.832 ± 0.060	0.891 ± 0.062	0.831 ± 0.046	0.837 ± 0.035
Ct.Ar [mm^2]	Inj	0.386 ± 0.025[abcd]		0.352 ± 0.029[d]	
	Non	0.455 ± 0.020	0.506 ± 0.025[ef]	0.391 ± 0.021	0.446 ± 0.035
Ct.Ar/Tt.Ar [%]	Inj	45.8 ± 3.9[abd]		41.1 ± 3.7[d]	
	Non	54.9 ± 3.2	57.1 ± 5.8[ef]	47.1 ± 2.0	53.3 ± 4.9
J [mm^4]	Inj	0.081 ± 0.007[bd]		0.078 ± 0.008[d]	
	Non	0.090 ± 0.011	0.105 ± 0.013[ef]	0.081 ± 0.008	0.089 ± 0.011

By ANOVA among both legs of BTX-treated groups, significant:

[a] Effect of *unloading*

[b] Effect of *BTX injection*

[c] *Unloading* × *BTX injection* interaction

[d] Paired *t*-test between uninjected and BTX-injected legs within group

By ANOVA among untreated controls and uninjected leg of BTX group, significant:

[e] Effect of *unloading*

[f] Effect of *BTX treatment*

[g] *Unloading* × *BTX treatment* interaction

+BTX BTX-treated groups, *−BTX* untreated controls, *Inj* injected leg, *Non* noninjected leg

of the BTX groups differed from their respective CON and HLU untreated controls (Table 2, $p < 0.01$).

Indirect BTX Effect and Body Mass

Since the BTX-treated groups experienced a decline in body mass while the CON and HLU groups did not, we used linear regressions to examine whether weight loss contributed to the indirect effects of BTX on bone and muscle outcomes. The percent change in body mass explained 38, 57, and 63 % of the variation in percent change in BMD, final Tb.BV/TV, and final gastrocnemius mass ($p < 0.0001$ for all), respectively. Notably, when *unloading, BTX treatment*, and percent change in body mass were all included as independent variables in the regression model, the model R^2 improved to >0.9 and *unloading* and *BTX treatment* were both significant predictors, whereas the effect of body mass did not remain significant. Therefore, the indirect effect of BTX injection is not completely explained by body mass changes.

Discussion

In this study, we generated an experimental model of extreme disuse by combining hind limb muscle paralysis and elimination of ground reaction forces to study musculoskeletal atrophy. We hypothesized that combining hind limb paralysis and unloading would have a greater deleterious effect on bone and muscle losses than either intervention alone. In support of our hypothesis, the combined HLU+BTX intervention had detrimental effects beyond that of either intervention alone for most measurements including hind limb BMD, trabecular bone volume fraction and thickness at the proximal tibia and distal femur, and midshaft tibial cortical bone area and thickness. Contrary to our initial hypothesis, the combination of HLU+BTX caused soleus atrophy equal to that of unloading alone and gastrocnemius atrophy equal to that of BTX alone.

However, these conclusions are limited by our observation of a marked indirect (systemic) effect of BTX treatment, manifested as lower BMD and worse bone architecture in the uninjected legs in both BTX-treated groups compared to their respective control groups with no exposure to BTX. Moreover, the indirect effects on hind limb BMD and tibial microarchitecture were nearly as profound as those of direct BTX treatment of the injected leg and often exceeded the effects of hind limb unloading alone (Fig. 7). These results were unforeseen given that other investigators encountered only minor and negligible indirect effects using the same BTX dose as that used here [9, 15]. Ultimately, it was difficult for us to evaluate the

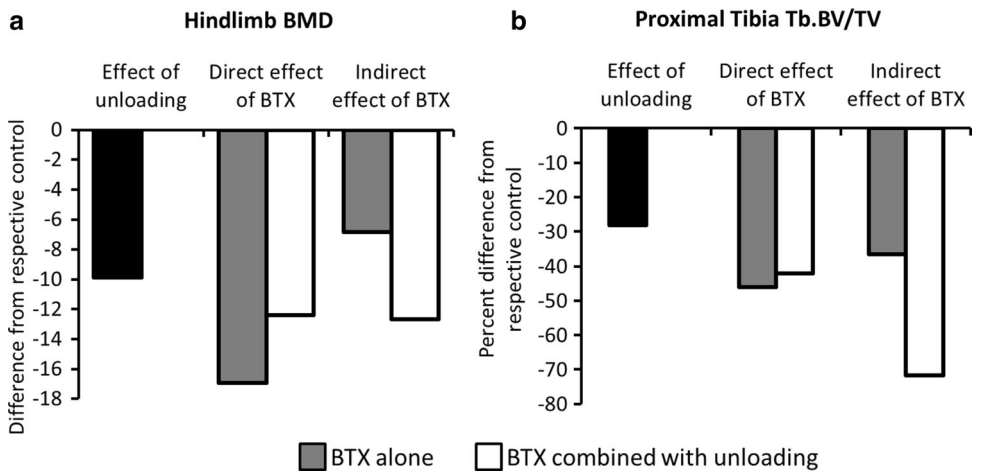

Fig. 7 Relative effect of unloading, direct BTX treatment, and indirect BTX treatment on **a** hind limb bone mineral density and **b** proximal tibia trabecular bone volume fraction. The magnitude of the effect of unloading (*black bars*) was calculated as the difference between average values of the HLU and CON groups. The direct effect of BTX alone (*shaded bars*) and BTX combined with unloading (*unshaded bars*) was calculated as the difference between the injected and contralateral limbs of the CON+BTX and HLU+BTX groups, respectively. The indirect effect of BTX alone and combined with unloading was calculated as the difference between the contralateral leg of the CON+BTX or HLU+BTX group and the untreated CON or HLU group, respectively

relative potency of muscle paralysis and unloading on bone and muscle since the indirect effects seen in the contralateral limb likely contributed to the changes seen in the BTX-injected limb.

The combined intervention used here was also recently studied by Warden et al. [14], though they carried out HLU for 6 weeks compared to our 3 weeks. Many of our results are similar to theirs, such as some additional skeletal effects of BTX-induced muscle paralysis when combined with hind limb unloading and a diminished effect of BTX treatment in HLU animals relative to BTX-injected cage controls. Importantly, however, Warden et al. did not include control groups without BTX injection, neither cage controls nor HLU mice, and thus could not account for possible indirect effects of BTX injection. Their conclusions might be reconsidered in light of the results of the current study demonstrating nonnegligible indirect effects with BTX treatment. For instance, Warden et al. state that HLU has a greater effect on bone than BTX treatment, which they conclude by comparing the contralateral leg of their HLU and BTX-treated group (subject to indirect effects that were not quantified) to the treated leg of the cage control group. We demonstrate that this reasoning may be flawed since our HLU group that did not receive a BTX injection fared better for every outcome than the cage controls with BTX treatment. In fact, our results point to an opposite conclusion to that of Warden et al., namely, that BTX-induced muscle paralysis is more deleterious to bone than HLU. As we hypothesized, the direct effect of BTX exceeded the effect of unloading on hind limb BMD (Fig. 7a), trabecular bone volume fraction (Fig. 7b) and thickness, and cortical area fraction and thickness.

In general, we found greater negative indirect effects of BTX treatment on muscle mass, bone mass, and bone morphology than previously reported in studies of unilateral BTX treatment in normally loaded animals [9, 15, 16, 26]. BTX-treated mice in the current study had a persistent deficit in body mass compared to the control group, which was also greater than that reported in past studies and may have contributed to the observed indirect effects on bone and muscle. The only major difference between the current study and prior work is our use of younger animals (11 weeks old rather than 15–16 weeks old). The BTX treatment might have been more debilitating for these late adolescent mice since they were still growing, albeit slowly, at this age [27]. Mice receiving both a BTX injection and HLU initially experienced early weight loss and lethargy despite the prophylactic addition of diet supplements and the 3-day interval between injection and unloading to foster accommodation to the paralysis. Lactated Ringer's solution was given immediately on day 1 when their moribundity was apparent, and recovery thereafter was swift. The CON+BTX group had less weight loss, was more active, and appeared to be in better health than the HLU+BTX group.

The ill health of the BTX-treated mice, though transient, likely contributed to the indirect effects. The decrease in body mass explained 38–63 % of the variation in musculoskeletal outcomes in the uninjected leg of the BTX-treated groups and their respective controls, accounting for much of the observed indirect effects. It is possible that a general reduction in activity in the BTX-injected groups could also have contributed to bone and muscle atrophy, particularly in the cage-control group, although we did not

quantify activity levels. Acute starvation with a concomitant metabolic acidosis is known to cause a decline in bone formation and stimulate calcium release directly from bone [28] in addition to muscle degradation [29]; thus, the effect of early body weight declines in the BTX-injected groups on bone and muscle could be explained by this mechanism. Finally, mice in the BTX groups were handled more because they were weighed more frequently and paralysis assessments were performed biweekly. This increased handling may have led to greater stress levels in the BTX groups and contributed to muscle atrophy and bone loss. Distant effects arising from the local BTX injection itself are also a possible, though less likely, contributor to the observed indirect effects as there is evidence of retrograde transport to the central nervous system [30], increase in mean jitter of distant muscles [31, 32], and cholinergic blockade [33]. There have not been any investigations into the influence of BTX on bone signaling pathways that would support a systemic effect. Since the indirect effect of BTX was an unexpected finding, this experiment was not designed to address its underlying mechanisms. Future studies should measure metabolic, adrenocortical, and physical activity to better understand the acute weight loss following BTX injection in adolescent mice.

As use of the BTX injection model of disuse in rodents increases, our results suggest it is important to bear in mind the potential for indirect effects that may confound the interpretation of how the elimination of muscle contractions influences bone adaptation. It is possible that the potent skeletal effects of BTX treatment involved mechanisms other than the direct action of muscle loading on bone. Moreover, the contralateral leg may not serve as an adequate control; and thus, groups of uninjected animals may need to be included as additional controls.

Our study was limited in that we only studied a single time point of 24 days post-injection to coincide with maximal muscle atrophy and bone loss [16], so we cannot discern whether the relative effects of HLU and BTX-induced paralysis and indirect BTX effects would be the same at earlier or later time points. Furthermore, we lacked in vivo longitudinal measurements of bone microarchitecture and muscle that would have allowed a more comprehensive examination of the rate and timing of bone and muscle changes. Additionally, it is important to note that BTX-induced paralysis leads to slightly reduced ground reaction forces [9, 12] (-11 to -23 %), so it is not purely a model of reduced muscle forces.

In conclusion, combining HLU and BTX injection resulted in the greatest musculoskeletal impairment, though the direct effect of BTX was diminished when combined with unloading. Administered individually, BTX-induced muscle paralysis appeared to have a greater detrimental effect on bone than HLU, but strong indirect effects on the uninjected legs of BTX-treated mice confounded our interpretation of the relative contribution of forces from muscle contraction versus external loading to skeletal health.

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Disclosures None

References

1. Judex S, Carlson KJ (2009) Is bone's response to mechanical signals dominated by gravitational loading? Med Sci Sports Exerc 41:2037–2043
2. Kohrt WM, Barry DW, Schwartz RS (2009) Muscle forces or gravity: what predominates mechanical loading on bone? Med Sci Sports Exerc 41:2050–2055
3. Robling AG (2009) Is bone's response to mechanical signals dominated by muscle forces? Med Sci Sports Exerc 41: 2044–2049
4. Frost HM (1997) On our age-related bone loss: insights from a new paradigm. J Bone Miner Res 12:1539–1546
5. Morey-Holton E, Globus RK, Kaplansky A, Durnova G (2005) The hind limb unloading rat model: literature overview, technique update and comparison with space flight data. Adv Space Biol Med 10:7–40
6. Tian X, Jee WS, Li X, Paszty C, Ke HZ (2011) Sclerostin antibody increases bone mass by stimulating bone formation and inhibiting bone resorption in a hind limb-immobilization rat model. Bone 48:197–201
7. Földes I, Gyarmati J, Rapcsák M, Szöör A, Szilágyi T (1986) Effect of plaster-cast immobilization on the bone. Acta Physiol Hung 67:413–418
8. Wagner EB, Granzella NP, Saito H, Newman DJ, Young LR, Bouxsein ML (2010) Partial weight suspension: a novel murine model for investigating adaptation to reduced musculoskeletal loading. J Appl Physiol 109:350–357
9. Warner SE, Sanford DA, Becker BA, Bain SD, Srinivasan S, Gross TS (2006) Botox induced muscle paralysis rapidly degrades bone. Bone 38:257–264
10. Tuukkanen J, Wallmark B, Jalovaara P, Takala T, Sjögren S, Väänänen K (1991) Changes induced in growing rat bone by immobilization and remobilization. Bone 12:113–118
11. Thompson DD, Rodan GA (1988) Indomethacin inhibition of tenotomy-induced bone resorption in rats. J Bone Miner Res 3:409–414
12. Manske SL, Boyd SK, Zernicke RF (2011) Vertical ground reaction forces diminish in mice after botulinum toxin injection. J Biomech 44:637–643
13. Gross TS, Poliachik SL, Prasad J, Bain SD (2010) The effect of muscle dysfunction on bone mass and morphology. J Musculoskelet Neuronal Interact 10:25–34

14. Warden SJ, Galley MR, Richard JS, George LA, Dirks RC, Guildenbecher EA, Judd AM, Robling AG, Fuchs RK (2013) Reduced gravitational loading does not account for the skeletal effect of botulinum toxin-induced muscle inhibition suggesting a direct effect of muscle on bone. Bone 54(1):98–105
15. Poliachik SL, Bain SD, Threet D, Huber P, Gross TS (2010) Transient muscle paralysis disrupts bone homeostasis by rapid degradation of bone morphology. Bone 46:18–23
16. Manske SL, Boyd SK, Zernicke RF (2010) Muscle and bone follow similar temporal patterns of recovery from muscle-induced disuse due to botulinum toxin injection. Bone 46:24–31
17. Aoki KR (2001) A comparison of the safety margins of botulinum neurotoxin serotypes A, B, and F in mice. Toxicon 39:1815–1820
18. Morey-Holton ER, Globus RK (2002) Hindlimb unloading rodent model: technical aspects. J Appl Physiol 92:1367–1377
19. Spatz JM, Ellman R, Cloutier AM, Louis L, van Vliet M, Suva LJ, Dwyer D, Stolina M, Ke HZ, Bouxsein ML (2013) Sclerostin antibody inhibits skeletal deterioration due to reduced mechanical loading. J Bone Miner Res 28:865–874
20. Ellman R, Spatz J, Cloutier A, Palme R, Christiansen BA, Bouxsein ML (2013) Partial reductions in mechanical loading yield proportional changes in bone density, bone architecture, and muscle mass. J Bone Miner Res 28:875–885
21. Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Müller R (2010) Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. J Bone Miner Res 25:1468–1486
22. Ellman R, Spatz J, Cloutier A, Palme R, Christiansen B, Bouxsein ML (2013) Partial reductions in mechanical loading yield proportional changes in bone density, bone architecture, and muscle mass. J Bone Miner Res 28(4):875–885
23. Hildebrand T, Ruegsegger P (1997) Quantification of bone microarchitecture with the structure model index. Comput Methods Biomech Biomed Eng 1:15–23
24. Hildebrand T, Rüegsegger P (1997) A new method for the model-independent assessment of thickness in three-dimensional images. J Microsc 185:67–75
25. Hildebrand T, Laib A, Müller R, Dequeker J, Rüegsegger P (1999) Direct three-dimensional morphometric analysis of human cancellous bone: microstructural data from spine, femur, iliac crest, and calcaneus. J Bone Miner Res 14:1167–1174
26. Manske SL, Boyd SK, Zernicke RF (2010) Muscle changes can account for bone loss after botulinum toxin injection. Calcif Tissue Int 87:541–549
27. Glatt V, Canalis E, Stadmeyer L, Bouxsein ML (2007) Age-related changes in trabecular architecture differ in female and male C57BL/6J mice. J Bone Miner Res 22:1197–1207
28. Grinspoon SK, Baum HBA, Kim V, Coggins C, Klibanski A (1995) Decreased bone formation and increased mineral dissolution during acute fasting in young women. J Clin Endocrinol Metab 80:3628–3633
29. Finn PF, Dice JF (2006) Proteolytic and lipolytic responses to starvation. Nutrition 22:830–844
30. Wiegand H, Erdmann G, Wellhoner HH (1976) 125I-labelled botulinum A neurotoxin: pharmacokinetics in cats after intramuscular injection. Naunyn Schmiedebergs Arch Pharmacol 292:161–165
31. Lange DJ, Rubin M, Greene PE, Kang UJ, Moskowitz CB, Brin MF, Lovelace RE, Fahn S (1991) Distant effects of locally injected botulinum toxin: a double-blind study of single fiber EMG changes. Muscle Nerve 14:672–675
32. Lange DJ, Brin MF, Warner CL, Fahn S, Lovelace RE (1987) Distant effects of local injection of botulinum toxin. Muscle Nerve 10:552–555
33. Dutton JJ (1996) Botulinum-A toxin in the treatment of craniocervical muscle spasms: short- and long-term, local and systemic effects. Surv Ophthalmol 41:51–65