Introduction

Bone development is arguably the most important aspect of skeletal physiology, as this process largely determines skeletal size and shape throughout life. Although it can be questioned whether a high ‘peak bone mass’ guarantees a strong skeleton for life, it is clear that changes in bone strength are much more rapid during skeletal development than later on1. Thus, any deficit in bone strength occurring during growth will be difficult to compensate for thereafter.

It is widely appreciated that mechanical factors are key determinants of bone strength development. The largest physiological loads on the skeleton result from muscle contractions, which put several-fold larger stresses on the skeleton than the simple effect of gravity2. It is therefore not surprising that disease processes which interfere with muscle development (e.g., muscle dystrophy, spina bifida, poliomyelitis) consistently have a negative effect on bone development3,4.

The authors have no conflict of interest.

Original Article

Effect of a single botulinum toxin injection on bone development in growing rabbits

F. Rauch and R. Hamdy

Genetics Unit, Shriners Hospital for Children, Montreal, Canada

Abstract

Intramuscular injections with botulinum toxin A (BTX-A) lead to a rapid decrease in muscle mass and force, but the effect of this drug on bone development is unclear. In the present pilot study we evaluated the effect of a one-time injection of BTX-A in growing rabbits. Twelve young (weight 1.5 kg) New Zealand rabbits were randomly assigned to receive either BTX-A (total dose 8 units per kg body weight) or sodium chloride 0.9% injections into the left quadriceps and gastrocnemius muscles. Both groups continued to gain weight in a similar manner following the injection. However, when the animals were sacrificed at five weeks after the injection, the group receiving BTX-A had a significant deficit (of 10%) in gastrocnemius muscle mass on the injected side, whereas no significant side-difference was found for the quadriceps. BTX-A injections did not affect the length of the tibia. Nevertheless, bone mineral content of the whole tibia, as measured by dual-energy X-ray absorptiometry, was 7% lower in the BTX-A injected side than on the contralateral side. Peripheral quantitative computed tomography showed that this bone mass deficit was larger in the metaphysis than in the epiphysis or diaphysis. In the diaphysis, the bone mass deficit was due to a reduction in cross-sectional bone dimensions, which equally affected the cross-section of the entire bone, the cortical compartment and the marrow space. BTX-A injections did not have a detectable effect on cortical bone mineral density. The bone mass deficit in the diaphysis thus appeared to be caused by a lack of periosteal bone apposition rather than increased endocortical or intracortical resorption. These preliminary data suggest that intramuscular BTX-A injections can have a deleterious effect on the development of bones that are loaded by the injected muscles.

Keywords: Botulinum Toxin A, Growth, Muscle, Periosteum, Rabbit

Original Article

Effect of a single botulinum toxin injection on bone development in growing rabbits

F. Rauch and R. Hamdy

Genetics Unit, Shriners Hospital for Children, Montreal, Canada

Abstract

Intramuscular injections with botulinum toxin A (BTX-A) lead to a rapid decrease in muscle mass and force, but the effect of this drug on bone development is unclear. In the present pilot study we evaluated the effect of a one-time injection of BTX-A in growing rabbits. Twelve young (weight 1.5 kg) New Zealand rabbits were randomly assigned to receive either BTX-A (total dose 8 units per kg body weight) or sodium chloride 0.9% injections into the left quadriceps and gastrocnemius muscles. Both groups continued to gain weight in a similar manner following the injection. However, when the animals were sacrificed at five weeks after the injection, the group receiving BTX-A had a significant deficit (of 10%) in gastrocnemius muscle mass on the injected side, whereas no significant side-difference was found for the quadriceps. BTX-A injections did not affect the length of the tibia. Nevertheless, bone mineral content of the whole tibia, as measured by dual-energy X-ray absorptiometry, was 7% lower in the BTX-A injected side than on the contralateral side. Peripheral quantitative computed tomography showed that this bone mass deficit was larger in the metaphysis than in the epiphysis or diaphysis. In the diaphysis, the bone mass deficit was due to a reduction in cross-sectional bone dimensions, which equally affected the cross-section of the entire bone, the cortical compartment and the marrow space. BTX-A injections did not have a detectable effect on cortical bone mineral density. The bone mass deficit in the diaphysis thus appeared to be caused by a lack of periosteal bone apposition rather than increased endocortical or intracortical resorption. These preliminary data suggest that intramuscular BTX-A injections can have a deleterious effect on the development of bones that are loaded by the injected muscles.

Keywords: Botulinum Toxin A, Growth, Muscle, Periosteum, Rabbit

Introduction

Bone development is arguably the most important aspect of skeletal physiology, as this process largely determines skeletal size and shape throughout life. Although it can be questioned whether a high ‘peak bone mass’ guarantees a strong skeleton for life, it is clear that changes in bone strength are much more rapid during skeletal development than later on1. Thus, any deficit in bone strength occurring during growth will be difficult to compensate for thereafter.

It is widely appreciated that mechanical factors are key determinants of bone strength development. The largest physiological loads on the skeleton result from muscle contractions, which put several-fold larger stresses on the skeleton than the simple effect of gravity2. It is therefore not surprising that disease processes which interfere with muscle development (e.g., muscle dystrophy, spina bifida, poliomyelitis) consistently have a negative effect on bone development3,4.

Despite the obvious clinical importance of the topic, experimental work on the functional muscle-bone unit during development is scarce. A number of experimental approaches have been used to evaluate the effect of decreased muscle forces on bone development, including casting, hindlimb suspension, nerve dissection and tenotomy5,6. However, the drawback of these methods is that they are quite invasive and that they do not specifically target muscle action.

A more selective inhibition of muscle function might be achieved by using Clostridium botulinum type-A neurotoxin (BTX-A). Its specificity for motor nerve terminals makes it an ideal induction agent for muscle weakness7. Following intramuscular injection, BTX-A induces flaccid paralysis of
F. Rauch and R. Hamdy: Botox and bone development

injected muscles by blocking neuromuscular transmission. In commonly used doses, the effects of BTX-A injections are strictly local and no systemic effects are noticed7. It has been shown in juvenile rats that muscle force decreases by more than 80% within a few days after a single BTX-A injection6. In adult rabbits, muscle force is decreased by 70% at four weeks after a BTX-A injection8. Studies in adult rats and mice have also shown that a single BTX-A injection into the quadriceps and gastrocnemius muscles leads to a decrease in bone mass at the tibia within a few weeks9,10. The effects of intramuscular BTX-A injections on the developing bone have not been assessed in any detail.

In the present pilot study, we therefore evaluated the effect of a one-time injection of BTX-A on bone development in growing rabbits. The experiment was designed to weaken two muscles that put loads on the tibia, the quadriceps and the gastrocnemius, and evaluate the influence of this intervention on the tibia. As is known from clinical observations, muscle weakness during growth is often associated with a reduced bone diameter4,5. We were therefore particularly interested in examining cross-sectional growth, which led to the choice of peripheral quantitative computed tomography as the main method to evaluate experimental results.

Materials and methods

Experimental design

Twelve skeletally immature, male New Zealand white rabbits (body weight 1.5 kg) were obtained and studied with the approval of the Animal Care Committee of McGill University, Montreal, Canada. Animals were housed individually in accordance with Canadian Council on Animal Care Guidelines. All animals were allowed normal activity in a cage 65 x 45 x 30 cm. The rabbits received a standard diet and water ad libitum. Animals were divided into two study groups, the BTX-A group and the control group. The BTX-A group received a one-time intramuscular injection of BTX-A into the left quadriceps and left gastrocnemius. The control group received injections of 0.9% sodium chloride solutions using the identical procedure and an equal injection volume as the BTX-A group.

BTX-A injection protocol

The BTX-A group received a one-time intramuscular injection of BTX-A into the quadriceps and the gastrocnemius of the left hindlimb. The total dose of BTX-A given to each of these animals was 8 units per kg body weight. A 100 unit vial of vacuum-dried BTX-A (BOTOX, Allergan, Inc., Toronto, Ontario, Canada) was reconstituted with 0.9% sodium chloride to a concentration of 20 units per ml. Prior to injection, rabbits were sedated with a subcutaneous injection of diazepam given at a dose of 5 mg per kg body weight. The anterior compartment of the thigh, containing the quadriceps musculature, was isolated by manual palpation. BTX-A was injected intramuscularly into the quadriceps with a 27-gauge needle. The injection sites were determined by visually dividing the quadriceps into superior and inferior halves, as described by Longino et al.9. Each half was further subdivided into medial, central, and lateral sections. One-seventh of the total BTX-A dose was injected into each of these six quadriceps sections. The remaining BTX-A was injected into the middle of the gastrocnemius muscle.

The control group received injections of 0.9% sodium chloride solutions using the identical procedure and an equal injection volume as the BTX-A group.
Measurement procedures

Following sacrifice, hindlimbs were dissected and wet muscle mass was determined for each hindlimb. The quadriceps and gastrocnemius muscles were weighed separately. The muscle masses were measured using a commercial scale with an accuracy of 0.01 g.

Immediately following dissection, tibia length was measured as the distance from the tibial plateau to the tibiotalar joint surface using a caliper. Dual X-ray absorptiometry was then performed using a Hologic Discovery A device (Hologic Inc., Waltham, MA, USA). Bone mineral content (BMC) of the entire tibia was determined using the manufacturer’s Small Animal Software package.

Peripheral quantitative computed tomography (pQCT) was performed using the Stratec XCT2000 equipment (Stratec Inc., Pforzheim, Germany). The scanner was positioned on the tibial plateau and a coronal computed radiograph (scout view) of the entire tibia was carried out. The scout view was used to determine the position of the measurement sites. The tibia was sampled at nine levels. The first measurement was performed at 2 mm distance from the tibial plateau (corresponding to the center of the epiphysis), the second in the metaphysis at the 10% site (the location where distance to the tibial plateau corresponds to 10% of tibial length). The remaining seven measurements were made on the diaphysis. These sections started at the 20% site and were spaced by 10% of tibial length.

At each measurement site, a single tomographic slice of 2.0 mm thickness was taken at a voxel size of 0.2 x 0.2 x 2 mm. The speed of the translational scan movement was set at 10 mm/s. Image acquisition, processing and the calculation of numerical values were performed using the manufacturer’s software package (version XCT 5.50D). The epiphyseal scan was assessed using the CALCBD algorithm at a threshold of 280 mg/cm3. Measures at the metaphysis and diaphysis were obtained at a threshold of 480 mg/cm3 using the software’s CORTBD routine. The following measures and definitions are used to present the results of the pQCT analyses:

- **Bone mineral content (BMC)**: The amount of mineral in a cross-sectional bone slice of 1 mm thickness.
- **Total cross-sectional area**: The surface area of the entire bone cross-section. This includes cortex and marrow space. This measure directly reflects changes in cross-sectional bone size through periosteal apposition.
- **Cortical cross-sectional area**: The surface area of the cortical bone cross-section. Marrow space is excluded. This measure is influenced by periosteal apposition and endocortical resorption or apposition.
- **Marrow cross-sectional area**: The surface area of the marrow space. This measure is determined by endocortical resorption or apposition.
- **Total bone mineral density (BMD, measured at the epiphysis)**: Volumetric BMD averaged across the entire bone cross-section. This is influenced by the relative contributions of the cortical and trabecular compartments and the densities within these compartments.

Statistical analysis

All outcome measures are reported as relative percentage deficits, comparing the treated left hindlimb to the untreated right hindlimb. The relative percentage deficits of the seven diaphyseal measurement sites were pooled to decrease variability of results and to facilitate reporting. Relative deficits were compared between the BTX-A and the control groups and tested for significance using Student’s t-test. Differences between results at the epiphysis, metaphysis and diaphysis were tested for significance using analysis of variance for repeated measures.

Results

The injection procedures were well tolerated by all rabbits. None of the animals displayed any specific signs of toxin overdose such as ptosis or respiratory distress. Although the gait pattern was not formally tested, BTX-A injected rabbits appeared to limp slightly during the first two weeks after the injections. Thereafter, there were no obvious group differences in the pattern of movement, which was limited to the cage area. Rabbits in the BTX-A and control groups gained weight at a similar speed throughout the study interval (Figure 1).

Tibia length was not affected by the BTX-A injections.
Figure 2. The BTX-A group had a muscle mass deficit in the left hindlimb, but the difference to controls was significant only for the gastrocnemius muscle. Dual-energy X-ray absorptiometry revealed that the tibia on the BTX-A injected side had a mineral mass deficit of about 7%.

This overall bone mass deficit was analyzed in more detail using pQCT (Figure 3). At the level of the epiphysis, the BTX-A group had a BMC deficit of 7% on the treated side (Figure 4). This was the result of (non-significant) deficits in both total BMD and bone cross-sectional area. In the metaphysis, the BMC deficit amounted to 19%. This bone mass deficit at the metaphysis was entirely due to a smaller cross-sectional area of the cortex, as no effect of BTX-A injections on cortical BMD was found. The BMC deficit in the BTX-A group was significantly larger at the metaphysis than at the epiphysis and the diaphysis ($p<0.001$).

At the diaphysis, a BMC deficit of 9% was found on the BTX-A injected side (Figure 4). Similar to the metaphysis, this was explained by the smaller cortical cross-sectional area. Cortical BMD was not affected by BTX-A. Both the total cross-sectional area and the marrow cross-sectional area were lower on the BTX-A injected side. The combined result of these changes was a 10% deficit in calculated torsional strength (Strength-Strain Index). The findings in the diaphysis are summarized graphically in Figure 5.

**Discussion**

In this study we found that a one-time injection of BTX-A into the quadriceps and gastrocnemius muscles had a
deleterious effect on bone development. Five weeks after the injection, the tibia on the injected side had lower mineral mass, both globally and in each anatomical subregion (epiphysis, metaphysis, diaphysis). Muscle mass was also lower in BTX-A injected hindlimbs, even though this was significant only for the gastrocnemius. However, there was no sign that BTX-A affected longitudinal bone growth. These results are certainly in accordance with the hypothesis that bone strength development is driven by muscle forces. However, other possibilities, such as a direct effect of BTX-A on bone, cannot be formally ruled out on the basis of the present data.

At first glance, our results are broadly similar to those obtained in adult mice, rats and rabbits, where locally decreased bone and muscle mass were found after BTX-A injections9-11. There are, however, clear differences between the present findings and those of these previous reports. Compared to the study on adult rabbits, the BTX-A induced decrease in muscle mass was much less in our animals (below 10%, compared to 36% in the adult rabbits), even though we used a somewhat higher dose of BTX-A. It is thus possible that our growing animals either did not lose as much muscle mass after the BTX-A injection, or that they recovered their muscle mass quicker than the adult rabbits.

As to the skeletal effects of BTX-A, there is a fundamental difference between the findings in adult mice and in the present study. In the study on adult mice, the lower bone mass on the BTX-A injected side resulted from a loss of bone from endosteal surfaces, whereas outer bone size was unaffected11. In our study, however, there was no indication of an excessive loss of endocortical bone. Quite to the contrary, marrow cross-sectional area was lower on the BTX-A injected side, showing that endocortical bone resorption was even diminished. The bone mass deficit in the BTX-A group was thus clearly explained by decreased periosteal apposition. This discrepancy between studies may reflect the differences in how adult and growing respond to a decrease in mechanical loading.

In the present study, the bone mass deficit was higher at the metaphysis than at the diaphysis or the epiphysis. This might be related to the fact that in growing bones the tissue at the metaphysis is younger than at the other two locations, as the metaphysis is the place where new bone is added through endochondral ossification. It is likely that the bone that was analyzed at the metaphysis was created after the BTX-A injections had been given. The epiphysial and diaphysial sites, however, probably contain a mixture of bone that was created before and after the injections. It is therefore not unexpected that the intervention had a larger effect at the metaphysis than at the other two sites.

This is a pilot study and thus has obvious limitations. First, our muscle analyses were limited to the determination of mass. In future experiments, it will be important to add tests of muscle function. Second, only a single time point was examined. It is therefore not possible to evaluate the dynamics of the changes following BTX-A injections. Third, bone histomorphometric measurements are needed to characterize the results in more detail.

In conclusion, these preliminary results suggest that a one-time injection of BTX-A has deleterious effects on local bone development. A more detailed characterization of this model is warranted.

Acknowledgements

The authors are supported by the Chercheur-Boursier Clinicien program of the Fonds de la Recherche en Santé du Québec. This study was supported by the Shriners of North America.

References