Introduction

Osteoporosis is a multifactorial disease characterized by a reduction in bone mass associated with an altered bone quality responsible for a loss in mechanical strength of bones leading to increasing risk of fracture. Disuse or prolonged bed rest are recognized conditions associated with a rapid bone loss in humans. In order to get a better understanding of the pathophysiology of disuse osteoporosis and the possible use of therapeutic strategies, animal models have been generated. Surgical models, such as amputation and denervation chordotomy lead to a rapid bone loss under the surgical lesion. However, because of confounding factors associated with surgical trauma, non-surgical techniques are now favored. Among them, hind-limb immobilization by cast application or tail suspension have been widely used. We have developed the BTX model using the Clostridium Botulinum toxin A to paralyze the Mus quadriceps femoris in the rat. A single intra-muscular BTX injection produces reversible skeletal muscle atrophy by blocking the presynaptic release of acetylcholine. The muscle loss becomes appreciable as soon as 1 week post injection and the associated bone reduction can be evidenced by dual energy X-ray absorptiometry (DXA), texture analysis of X-ray images or microcomputed tomography.
MicroCT analysis was performed on tibias with a SkySCAN 1172 microtomograph (Bruker-Skyscan, Kontich, Belgium) equipped with an X-ray tube working at 69 kV/100 μA. An
isotropic voxel size was fixed at 13.4 μm, the rotation step at 0.25° and exposure was performed with a 0.5 mm aluminum filter. Volumetric bone mineral density (vBMD) measurements were performed according to the manufacturer recommendations by using calibrated phantoms adapted to rat bones\(^9\). The CTAn Software (Skyscan, release 1.10.1.0) was used to measure the bone mass of the tibia (upper metaphysis). The first image selected for analysis was located just under the growth plate and then extended on 300 sections in the tibia. The volume of interest (VOI) was designed by interactively drawing a polygon on each 2D section. Only a few number of polygons need to be drawn (e.g. on the first section, several at the middle, and on the final section) since a routine facility calculated all the intermediary masks by interpolation. A first VOI (located at the same bone regions than qXRI) containing only trabecular bone and marrow cavity was drawn and a global threshold was used to select the trabeculae\(^20\). The trabecular bone volume (BV/TV, in %) representing the percentage of the cancellous space occupied by trabecular bone was determined. This VOI was also used to measure the trabecular vBMD (in g/cm\(^3\)). Another VOI comprising only cortical bone was obtained at the midshaft of each bone to determine cortical vBMD. The following microarchitectural descriptors of cortical bone: cortical thickness (Ct.Th, in μm), cortical area (Ct.Ar, in mm\(^2\)) and cross-sectional moment of inertia (CSMI, in mm\(^4\)) were measured with a lab-based routine made with ImageJ according to guidelines and nomenclature proposed by the American Society for Bone and Mineral Research\(^21\).

**Dual energy X-ray absorptiometry (DXA)**

*Ex vivo* DXA scans were performed with a Hologic discovery W (Hologic Inc, Bedfor, MA) using the APEX software in the high resolution small animal setting (release 3.3, Hologic Inc). Tibias were placed in a plastic jar filled with water (1 cm thickness) and with a mask drawn at the bottom to ensure reproducible positioning of bones. The densitometer was operated at 140/100 kV with a pixel resolution of 700 μm. The proximal metaphysis was used to evaluate trabecular bone by selecting a ROI up to 4mm from the growth plate. The midshaft tibia was used to measure cortical bone by applying a 4-mm ROI at the center.

**Three-point bending**

Three-point bending experiments were performed on the tibias. Before mechanical testing, bones were rehydrated in saline for 24 hrs at room temperature as described elsewhere\(^22\). Three-point bending strength was measured with a constant span length of 20 mm. The press head as well as the two support points were rounded to avoid shear load and cutting. Bones were positioned horizontally with the anterior surface facing upward, centered on the support and the pressing force was applied vertically to the midshaft of the bone. Each bone was tested with a loading speed of 2 mm/min\(^{-1}\) until failure with a 500N load cell on an Instron 5942 device (Instron, Elancourt, France). The load-time curve obtained was converted into a load-displacement curve by the Bluehill 3 software (Instron). Ultimate load and ultimate displacement were respectively defined as the maximum load and maximum displacement recorded before breakdown of the bone. Stiffness was calculated as the slope of the elastic deformation of the bone. The total absorbed energy was defined as the total area under the load-displacement curve and represented the total energy absorbed by the midshaft femur. Intrinsic parameters were calculated according to previous published equations\(^23,24\).
Quantitative backscattered electron imaging (qBEI)

Quantitative backscattered electron imaging was employed to determine the bone mineral density distribution (BMDD) in tibia cortical bone as previously reported $^{25,26}$. This methodology has been described in full details elsewhere $^{26}$. After 3-point bending, the upper tibia extremities were embedded undecalcified in poly (methylmethacrylate). Blocks were polished to a 0.5-μm finish with diamond particles, carbon-coated and observed with a scanning electron microscope (EVO LS10, Carl Zeiss Ltd, Nanterre, France) equipped with a five quadrants...
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The semi-conductor backscattered electron detector. The microscope was operated at 20 keV with a probe current of 250 pA and a working distance of 15 mm. The cortical bone area located at the mid-diaphysis was imaged at a 100X nominal magnification, corresponding to a pixel size of 1 μm per pixel. Six images per samples were taken and the gray levels distribution of each image was analyzed with a lab-made routine in ImageJ. Three variables were obtained from the bone mineral density distribution: Ca_{peak} as the most frequently observed calcium concentration, Ca_{mean} as the average calcium concentration and Ca_{width} as the width of the histogram at half maximum of the peak. The mineralized cortical mass (MCM) was calcu-

Figure 4. Quantitative X-ray imaging and correlation with microarchitectural and biomechanical parameters. (A) Trabecular and (B) cortical absorbing material density (AMD) in non- and BTX-injected side. Both parameters were significantly lower in the BTX-treated side. **: p<0.01 vs. saline-injected side. Regression analyses between (C) trabecular AMD and trabecular bone volume (BV/TV), cortical AMD and (D) cortical area, (E) cortical thickness, (F) bending stress, (G) ultimate strain, (H) bending modulus, (I) work to fracture, (J) Ca_{mean} and (K) MCM. Color code: blue= saline-injected side and red= BTX-injected side; NS= non-significant.
lated in order to account for modification in the degree of mineralization and cortical mass. This parameter for a given i animal was calculated as follow:

\[ MCM(i) = \frac{Ct.Ar(i) \times Ca_{mean}(i)}{Ca_{mean-group}} \]

where \( Ct.Ar(i) \) is the cortical area of the i animal, \( Ca_{mean}(i) \) is the mean calcium concentration of the i animal and \( Ca_{mean-group} \) the mean calcium concentration of all animals from the right (BTX-injected) or left (saline-injected) hindlimb group.

**Statistical analysis**

Statistical analysis was performed using the Systat statistical software release 13.0 (Systat Software Inc., San José, CA). All data were expressed as mean±standard error of the mean (SEM). Differences between groups were analyzed by a non-parametric Kruskal-Wallis test. Differences were considered significant when \( p<0.05 \).

**Results**

**Assessment of bone microarchitecture, mechanical response and mineralization properties in the BTX rat**

In order to ensure that BTX injections led to deteriorations of trabecular microarchitecture, we assessed trabecular parameters by microCT (Figure 2). As reported Table 1, BTX-injected sides presented with a significant 17% reduction in BV/TV as compared with saline-injected sides \( (p<0.001) \). Cortical bone microarchitecture was also investigated and a significant but moderate reductions were evidenced in Ct.Ar (-5.1%) and Ct.Th (-5.4%) in the BTX-injected hindlimb \( (p=0.04 \text{ and } p=0.003, \text{ respectively}) \). None of the other cortical parameters were significantly altered in BTX-injected hindlimb.

Mechanical response of the cortical bone was assessed at the midshaft tibia by 3-point bending. As reported Table 2, only a significant reduction in the work to fracture (-19%) was observed in BTX-injected hindlimb as compared to the saline-injected side. None of the other parameters (extrinsic and intrinsic) were significantly different between the two sides. Furthermore, the bone mineral density distribution was altered in the BTX-injected side with significant reductions in \( Ca_{peak} \) and \( Ca_{mean} \) by 5% and 5%, respectively.

**Assessment of bone mineral density by qXRI, DXA and microCT**

**qXRI**

The absorbing material density (AMD) was determined at the proximal tibia metaphysis (trabecular AMD) and tibia midshaft (cortical AMD). The aspect of the femur and tibia of a BTX rat appear on Figure 3. Quantitative results are depicted on Figure 4A and 4B. Trabecular and cortical AMDS were significantly lower by 20.6% and 4.8% in the BTX-injected as compared with the saline-injected side \( (p<0.001 \text{ and } p=0.007, \text{ respectively}) \). A good correlation between trabecular AMD and BV/TV was observed \( (R=0.804, p<0.001) \). On the other hand, cortical AMD was not correlated with neither Ct.Ar nor Ct.Th. Cortical AMD was linked to mechanical properties with significant correlations with bending stress \( (R=0.494, p=0.007) \) and bending modulus \( (R=0.735, p<0.001) \). On the other hand, no correlations were evidenced between cortical AMD and ultimate stress, work to fracture and \( Ca_{mean} \).

However as the cortical bone mass and degree of mineralization were altered in the BTX-injected site, we assessed whether cortical AMD could be linked to MCM, a parameter accounting for cortical area and degree of mineralization with qBEI (Figure 5). And indeed, cortical AMD was significantly associated with MCM \( (R=0.361, p<0.05) \).
DXA

Trabecular and cortical aBMD were determined at the proximal tibia metaphysis (trabecular) and tibia midshaft (cortical) (Figure 6). Trabecular aBMD was significantly reduced by 18% in the BTX-injected side as compared to the other hindlimb (p<0.001). At the cortical site, aBMD was significantly reduced by 8.9% in the BTX-injected side (p=0.007). Trabecular aBMD was significantly correlated with BV/TV (R=0.816, p=0.01). Cortical aBMD was significantly correlated with Ct.Ar (R=0.633, p<0.001), Ca.mean (R=0.527, p=0.01) and MCM (R=0.732, p<0.001). On the other hand, no significant correlations were found between cortical aBMD and the other investigated parameters, especially mechanical parameters.
Trabecular and cortical vBMDs were significantly lowered by 21% and 4.9%, respectively in BTX- compared with saline-injected sides (p<0.001 and p=0.003, respectively) (Figure 7). Trabecular vBMD was significantly correlated with BV/TV (R=0.886, p<0.001). Cortical vBMD was found associated to Ct.Th (R=0.443, p=0.02), Ct.Ar (R=0.404, p=0.03) and MCM (R=0.518, p=0.01). None of the other investigated parameters including mechanical parameters was significantly correlated to cortical vBMD.
Trabecular AMD was significantly correlated with trabecular BMD, BMC and vBMD

Trabecular AMD was significantly correlated with trabecular aBMD (R=0.802, p<0.001) and trabecular vBMD (R=0.842, p<0.001) (Figure 8). On the other hand, no correlations were found between cortical AMD, cortical aBMD and cortical vBMD.

Discussion

Osteoporosis is a disease characterized by alterations of bone mass and quality leading to an increase risk of bone fracture. Different factors can result in osteoporosis and among them disuse is recognized as a contributing factor leading to a rapid bone loss in human and animals.

In the present study, we used a validated model of disuse-osteoporosis caused by the injection of Botulinum toxin and a subsequent paralysis of the Mus quadriceps femoris. This model has been fully characterized previously by us and other groups in term of bone microarchitecture. Here we report, as expected, significant alterations of trabecular and cortical bone masses (-17% in BV/TV, -5.1% in Ct.Ar and -5.4% in Ct.Th).

Unexpectedly, despite these alterations in cortical microarchitecture, the mechanical resistance of cortical bone as assessed by 3-point bending was only moderately reduced in the BTX-injected hindlimb and only a lower work to fracture was evidenced. These findings are in agreement with the observations of Sheng et al. or more recently Grubbe et al.. Indeed, these authors reported, using Sprague Dawley or Wistar rats, that the mechanical resistance of BTX animal was only moderately affected at mid-diaphysis with a significant reduction in ultimate load. In the present study, we observed a significant reduction in the work to fracture which, as defined by Ritchie et al., is a measure of bone toughness. However, very little was known about the quality of the bone matrix in their animal model and as such in order to ascertain whether the mineralization was hampered, we evaluated the bone mineral density distribution by qBEI. We evidenced a significant reduction in the mean and peak calcium concentrations by 5%, that could potentially be responsible for the reduction in work to fracture as reported above although the mechanisms leading to such observation have yet to be determined.

Another aim of this study was to determine whether qXRI could be a validated method to highlight change in trabecular and cortical bone masses in rats and how it performs compared to DXA and high-resolution microCT. All these three methodologies are based on the absorption of X-rays by the bone mineral. As expected, trabecular AMD, aBMD and vBMD were significantly reduced in the BTX-injected side in the same order of magnitude. Additionally, all three parameters correlated well with BV/TV (R>0.8). On the other hand, cortical AMD failed to correlate with cortical microarchitecture whilst...
cortical aBMD and vBMD did. Surprisingly, cortical AMD and vBMD failed to correlate with the mean calcium concentration (Ca mean) whilst aBMD did. However, all three parameters were correlated with the Mineralized Cortical Mass, a parameter introduced in this study to account for reduction in cortical bone mass and mineralization of this envelope. It is worth noting that the order of correlation with MCM was aBMD>vBMD>AMD.

As said above, the three methodologies are based on the absorption of an incident X-ray beam by the bone mineral. However, the energy of the X-ray beam is different between the three methodologies as X-rays were generated at different accelerating voltage. Indeed, it is with DXA that X-rays were the most energetic followed by microCT and finally qXRI. This is important as high energy X-rays are less absorbed by soft material such as skin, muscle or even bone marrow and are high enough not to be totally absorbed by the bone mineral. The low accelerating voltage used for qXRI makes this methodology useable only in small animal models such as mouse or rat models.

An intriguing result was that only cortical AMD correlated with several 3-point bending parameters. The assumption that qXRI correlated well with mechanical resistance of the bone had been postulated before by Bassett et al. However, at this point, we do not have explanation to understand the failure of aBMD and vBMD to correlate with mechanical parameters.

qXRI was also performed by other research groups, but the way results are reported are slightly different although the experimental setups are very similar. However, although these authors used a similar setup to perform qXRI, the interpretation of the data is either made by comparing cumulative frequency histogram or calculating a mean gray level. In the present study, we have improved the detection method by taking into account the length of the bone tissue crossed by the X-ray beam. This allowed us to convert the mean gray level into the absorbing material density expressed in g/cm³ of material. It was not the case with the BTX model, but it is important to take into account the length of the bone crossed by the X-ray beam in situations where the outer bone diameter is affected.

In conclusion, bone quality is altered in the BTX- model of disuse at the microarchitecture level but also in the mineral component of the bone matrix. Furthermore, qXRI and the resulting AMD appeared informative in terms of trabecular bone and compared very well to other validated methodologies such as DXA and microCT. However, although qXRI may present several limitations in the assessment of cortical bone mass, this methodology correlated well with mechanical parameters.

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