RAMAN SPECTROSCOPY OF OSTEOPOROTIC RAT TIBIA
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Abstract
Fractures are the most frequent health problem associated with bone. Metabolic diseases, such as osteoporosis, affect skeletal integrity, reduce strength and toughness of bone and lead to increased risk of fragility.

In the present work, changes in the amount and/or quality of bone were studied in osteoporotic tibiae from female wistar rats compared to healthy controls [restudied samples of Ref. 1, 2 according to parameters of Ref. 3]. Osteoporosis was induced through ovariectomy. Bone composition and quality was evaluated employing Raman Spectroscopy. Several spectra were recorded. The height of the primary phosphate band (PO₄³⁻, ν₁) for the mineral at 959 cm⁻¹, the carbonate peak at 1070 cm⁻¹ under the combined phosphate-carbonate envelope 1010-1100 cm⁻¹ spectral range, the matrix bands at 855 cm⁻¹ (hydroxyproline), 875 cm⁻¹ and 920 cm⁻¹ (proline), as well as the three major peaks under amide I envelope (1620-1710 cm⁻¹) were measured after proper baselinining and deconvolution.

The mineral to matrix ratio [959 cm⁻¹ / (855 cm⁻¹ + 875 cm⁻¹ + 920 cm⁻¹)] was reduced, suggesting decreased mineral quantity in the osteoporotic tibiae compared to controls. Carbonate levels remained stable which implies absence of new bone tissue formation, though bone is known to follow a constant renewal procedure.

The mineral to amide I envelope ratio exhibited an increasing trend suggesting that amide I cannot be used as collagen metrics as it is subject to polarization effects. Further analysis of the amide I envelope shows that the band changes shape following bone disease, which is a result of the change in the ratio of the peaks lying under the amide I envelope. Therefore, changes in collagen cross-linking accompany reduction of mineral amount and lead to reduced strength and increased fragility in osteoporosis.

Introduction
Bone fracture is a major health issue due to significant financial burden apart from pain and severe mobility problems caused to fracture patients, especially to the elderly. A number of bone abnormalities can be accused for. Osteoporosis is a metabolic disorder directly related to bone fragility. It is associated with imbalance between bone formation from osteoblasts and resorption from osteoclasts in favor of the latter. Bone quantity is overall reducing with subsequent decline in skeletal integrity.

Bone mineral density (BMD) is a parameter measured in everyday clinical practice to evaluate bone condition and predict fracture. However, due to the
nature of the measurement, BMD is sensitive only to mineral changes while alterations in organic matrix are not detectable [4].

The mechanical properties of bone tissue depend both on the mineral and the matrix (primarily type I collagen fibrils) constituents. Although bone mineral density (BMD) has been extensively used as a predictor of bone fragility, it is becoming increasingly clear that it is not the sole determining factor. Knott et al [5] suggested that osteoporosis in avian bones is certainly not just a simple loss of apatite and collagen but involves significant changes in the biochemistry of the collagen molecule and consequently in the physical properties of the fiber.

The intermolecular cross-linking of bone collagen is a chemical feature that is intimately related to the way matrix collagen molecules are arranged in fibrils and provides fibrillar matrices with important mechanical properties such as tensile strength and viscoelasticity [6].

In this study, Raman Spectroscopy was used to evaluate decline in bone quality due to osteoporosis in ovariectomized female wistar rat tibia, concerning mineral loss and matrix cross-linking.

Materials and Methods
Left tibia from control and osteoporotic female wistar rats were used for the analysis [restudied samples of Ref. 1,2 according to parameters of Ref. 3]. Osteoporosis was induced through ovariectomy and confirmed by histological examination. Total density measurements by pQCT analysis were used as reference for all tibia. Biochemical marker (NTx and osteocalcin) levels in serum were also recorded for comparison. All necessary details are quoted in Ref. 2.

Several FT-Raman spectra (Bruker, FRA-106/S) were collected from different spots of tibia periosteum in the mid-diaphysis region. A laser line at 1064 nm (Nd:YAG laser) was used for excitation. The Rayleigh line was removed by means of a secondary filter and the scattered light was collected at an angle of 180°. The system was equipped with a liquid N₂ cooled Ge detector (D 418). The power of the incident laser line was about 370 mW on the sample’s surface. Typical spectral line width was 0.5 cm⁻¹ while the recorded spectra were the average of 300 scans.

Raman band parameters (height and area) of the characteristic mineral and matrix peaks were measured after proper baselining, curve fitting to Gaussian-Lorentzian peak profiles and deconvolution (fig. 1) using the Peakfit software (Peakfit© v4.0, Jandel Scientific, San Rafael, CA). To ensure reproducibility, baseline correction was performed using a two-point model at fixed band positions.

Bone compositional data were obtained using spectral data from the characteristic peaks of mineral and matrix: The primary phosphate ($v_1$) at 959 cm⁻¹, the carbonate peak at 1070 cm⁻¹ under the combined phosphate-carbonate envelope 1010-1100 cm⁻¹ spectral range and the bands at 855 cm⁻¹
(hydroxyproline), 875 cm\(^{-1}\) and 920 cm\(^{-1}\) (proline) for collagen. The proline and hydroxyproline bands were preferred as collagen metrics due to their lack of sensitivity to fibrillar polarization [7].

Figure 1. Raman spectrum of wistar rat tibia. Characteristic bands are appointed

Figure 2. Deconvolution of bands under phosphate envelope (A), carbonate envelope (B) and amide I envelope (C)

The amide I envelope (1620-1710 cm\(^{-1}\)) was also deconvoluted to reveal the peaks underneath. The peaks at 1660 and 1690 cm\(^{-1}\) were used in order to evaluate the cross-linking and subsequent quality of collagen [8].
The mineral to matrix ratio (MMR) \[959 \text{ cm}^{-1} / (855 \text{ cm}^{-1} + 875 \text{ cm}^{-1} + 920 \text{ cm}^{-1})\], the carbonate to phosphate (CPR) \[1070 \text{ cm}^{-1} / 959 \text{ cm}^{-1}\] as well as the \[1660 \text{ cm}^{-1} / 1690 \text{ cm}^{-1}\] ratio were used as Raman metrics to evaluate bone quality [3,7].

Results and Discussion

Mineral to Matrix ratio

The mineral to matrix ratio for the ovariectomized rats was reduced compared to the respective value for the control. This suggests that the amount of mineral compared to the matrix diminishes in osteoporotic bones, which is in agreement with the perception of osteoporosis that «calcium is lost in bone». It is also in consistence with the reduction of the total density measurements (pQCT) and the increase of the biochemical markers NTx and osteocalcin in blood serum for the same samples, quoted in Refs 1,2. However, the decrease was more pronounced for the last group which was sacrificed 145 days after ovariectomy. All data are quoted in Table 1.

<table>
<thead>
<tr>
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<th>Mineral to Matrix ratio</th>
<th>1660 cm(^{-1}) / 1690 cm(^{-1}) band area ratio</th>
<th>Carbonate to Phosphate ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.83 ± 0.60</td>
<td>1.75 ± 0.39</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>60 days after ovariectomy</td>
<td>7.76 ± 0.66</td>
<td>1.76 ± 0.48</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>145 days after ovariectomy</td>
<td>6.68 ± 0.96</td>
<td>2.52 ± 0.51</td>
<td>0.23 ± 0.01</td>
</tr>
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Sixty (60) days after ovariectomy, NTx and osteocalcin levels in serum have doubled [1,2]. At the same time, MMR remained stable (table 1). 145 days after ovariectomy, osteocalcin increased at approximately the same rate, while NTx value has immensely risen [1,2]. MMR for the same period of time, was reduced. It can be concluded that mineral to matrix ratio can be informative in cases where there is relative change between mineral and matrix but it cannot be of much value in increased bone turnover where the two components are removed with approximately the same rate.

On the other hand, the collection of information for bone state with Raman spectroscopy is rather advantageous as it highly localized, coming from a spot of a few microns in diameter on sample’s surface. Osteoporosis has also a local rather than bulk character as reduction in bone characteristics are not the same from point to point. In the areas with the higher degree of damage, fracture may easily take place. On the other hand, density measurements are averaged in the bone sections tested each time. NTx and osteocalcin levels in serum are actually a not direct measurement of bone metabolism and correspond to an average loss of bone imbalance between bone growth and resorption.
Carbonate to Phosphate ratio
Carbonate to phosphate ratio did not change significantly between the groups suggesting that there is no new bone growth at least at the spots where spectra where collected (Table 1) [3,7].

Amide I
Further analysis of the amide I envelope shows that the band changes shape following bone disease, which is a result of the change in the ratio of the peaks lying under the amide I envelope. Calculation of the ratio [1660 cm$^{-1}$ / 1690 cm$^{-1}$], which has been reported to refer to the non-reducible / reducible collagen crosslinking [8,9], reveals a considerable increase for osteoporotic samples compared to control. This can be attributed to increase in the amount of the non-reducible cross-links due to transformation of the reducible, and/or subsequent decrease of the reducible or less formation of them. The increase is more pronounced for the last group (145 days after ovariectomy). Therefore, changes in collagen cross-linking accompany reduction of mineral amount and lead to reduced strength and increased fragility in osteoporosis. These results are in agreement with the changes in collagen cross-linking as a result of age and osteoporosis monitored with FTIR and HPLC [8,10]. Raman spectra and amide I band envelope reveal that the non-reducible (trivalent) cross-linking is increased, meaning that the remaining collagen fibers are tied more strongly with each other. Thus, collagen network becomes stiffer and more rigid and loses its elasticity.

Conclusions
Left tibia from osteoporotic female wistar rats were studied with Raman spectroscopy in comparison with sex- and age-matched controls. Mineral to matrix ratio calculated from Raman spectra was found to decrease due to considerably increased collagen turn over compared to mineral dissolution as revealed by NTx and osteocalcin levels in serum for the same samples (quoted in reference 1,2). A substantial increase of the non-reducible cross-links was also detected which suggests that collagen network gets stiffer and less elastic due to denser connective bonds.

Raman spectroscopy can be used to monitor osteoporosis and the relevant quantity reductions in mineral and organic constituents of bone. It should be noted, though, that spectral data are sensitive to abnormal relative changes between them i.e. a higher rate of collagen removal than the rate of mineral during bone turn over. On the other hand, RS can detect biochemical alterations in collagen which also contribute to the deterioration of mechanical properties of bone. It can, conclusively, perform subsidiarily and complementarily to the current clinical tools for osteoporosis.

References

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