Comparison of Bone Quality in Healthy Male and Female Animal Models Using Raman Spectroscopy

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Abstract

It is a general perception that, women, especially the elderly, are more susceptible to bone diseases such as osteoporosis and prone to cracks than men. In the present work, the quality of bone, of female anile healthy animal models were evaluated by Raman spectroscopy and compared to male age-matched controls.

A group of three pairs of wistar rats (six animals) were sacrificed at the age of 13 weeks. Thigh and tibia were used for the analysis. Several spectra from periosteum were collected using a micro-Raman spectrometer. After appropriate deconvolution and band fitting, the intensity of the primary phosphate band ($\text{PO}_4^{3-}$, $v_1$) at 959 cm$^{-1}$, of 1072 cm$^{-1}$ for the carbonate of the mineral, the matrix bands at 855 cm$^{-1}$ (proline), 875 cm$^{-1}$ (hydroxyproline), as well as the 1668 cm$^{-1}$ and 1685 cm$^{-1}$ bands under the amide I envelope (1590-1710 cm$^{-1}$) were measured. The following Raman metrics were calculated: mineral to matrix ratio (MMR), mineral carbonation, mineral crystallinity and [1668 cm$^{-1}$ / 1685 cm$^{-1}$] which corresponds to collagen secondary structure.

It was shown that no statistically significant discrepancy exists between male and female in case where no disease has set in. The fact that female osteoporotic incidents are more frequent cannot be attributed to defective female bone.

1. Introduction

Older bone appears to be more sensitive to damage than younger bone. In addition age affects mechanical, structural and compositional properties of the bone. Osteoporosis is a disease in which the density and quality of bone are reduced. As bones become more porous and fragile, the risk of fracture is greatly increased. The loss of bone occurs silently and progressively. Often there are no symptoms until the first fracture occurs.

Bone is a composite material. It consists of a mineral part (a biological analogous of hydroxyapatite) embedded in collagen type I fibers. Bone mineral density (BMD) is a parameter measured in everyday clinical practice to evaluate bone condition and predict fracture[1].

The mechanical properties of bone depend in part on the chemical and structural aspects of its mineral phase. The mineral crystals in bone are similar to the geologic mineral hydroxylapatite, but typically contain numerous ionic substitutions. Bone mineral, which is one kind of bioapatite, is less crystalline than its geologic counterpart. The specific composition and low degree of crystallinity of bioapatite enhance its reactivity and probably account for its changes with aging and drug treatment[2].

Bone is a hierarchically structured composite which at the nanometer range can be described as a combination of a stiff inorganic mineral phase of carbonated apatite together with a softer organic phase (principally type I collagen, with a small amount of proteoglycans and noncollageneous proteins). The collagen forms $\approx$100- to 200-nm-diameter fibrils, with thin elongated mineral platelets inside and on the surface. These mineralized fibrils are then arranged into higher levels of structural motifs such as fibril arrays and lamellae[3].

Raman spectroscopy is a vibrational spectroscopy technique used to assess scattered light from biologic molecules and ions. Raman scattering occurs when molecules within a specimen are excited by incident laser light. Vibrational motions within the molecules lead to a small fraction of the light (approximately one in 107 photons) losing energy and being scattered at longer wavelengths [4]. The wavelength difference between scattered and incident light corresponds to molecular vibrations and leads to bands at characteristic frequency shifts in the Raman spectrum.
The purpose of the study was to compare healthy male and female rats in order to examine how sex differences can affect bone material characteristics. Although we know tissue age can affect bone material characteristics like mineral/matrix ratio, mineral maturity/crystallinity, relative pyridinoline collagen cross-link content, relative proteoglycan, and relative lipids content in trabecular bone of children and young adults[5], it is not well established the role of sex in affecting bone quality.

2. Materials and Methods

Tibia and thigh bones from three pairs of healthy wistar rats were used for analysis. For the Raman study six rats were sacrificed at the age of 13 weeks. The soft surrounding tissue of each bone was mechanically removed by scalpel (Fig. 1) and the bones were stored under standard conditions of temperature (−20°C). Eight micro-Raman spectra were collected from different spots of tibia and femoral periosteum. A characteristic micro-Raman spectrum taken from a spot on a sample can be seen in Fig. 2A and B. Four regions of interest were isolated for further analysis: the proline-hydroxyproline (830–900 cm⁻¹), the major phosphate (900–990 cm⁻¹), the combined carbonate–phosphate (1010–1130 cm⁻¹) and the amide I (1590–1710 cm⁻¹).

Baseline correction, for every spectral region separately, was performed using a two-point model. Local minima were chosen, at both sides, within a fixed (small) range of wavenumbers to ensure reproducibility. Major sub-bands were revealed employing the second-derivative approach. The proline-hydroxyproline spectral region contained bands at 875, 855 cm⁻¹ (Fig. 3A), while the phosphate envelope (900–990 cm⁻¹) was centering at 959 cm⁻¹ (Fig. 3B) in agreement with data quoted in literature [6]. Carbonate-phosphate envelope analysis revealed the presence of a major peak at around 1072 cm⁻¹ (Fig. 3C) [7]. Finally, the amide I spectral area included four major sub-bands at 1688, 1667, 1639 and 1609 cm⁻¹ (Fig. 4). Analysis included curve-fitting of the sub-bands to unconstrained Gaussian-Lorentzian profiles and deconvolution was performed using the Peakfit software (Peakfit© v4.0, Jandel Scientific, San Rafael, CA) and Origin 8.0. Raman intensities (heights) were measured.
Bone compositional data were obtained using spectral data from the characteristic bands of mineral and matrix: For the mineral to matrix ratio (MMR) the primary phosphate ($v_1$) at 959 cm$^{-1}$ and the bands at 855 cm$^{-1}$ (proline) and 875 cm$^{-1}$ (hydroxyproline) for collagen were used [$959$ cm$^{-1}$/$(855$ cm$^{-1} + 875$ cm$^{-1})$] due to their insensitivity to collagen cross-linking modifications [8,9,10,11]. Furthermore, the bands at 1668 and 1685 cm$^{-1}$ were used [1668 cm$^{-1}$/1685 cm$^{-1}$] in order to evaluate the cross-linking and subsequent quality of collagen [12]. The carbonate band at 1072 cm$^{-1}$, under the combined phosphate-carbonate envelope 1010–1130 cm$^{-1}$ spectral range, was used for the carbonate to phosphate ratio (CPR) [1, 9, 10, 11, 13]. The presence of a prominent carbonate band around 1072 cm$^{-1}$ in the Raman spectrum (Fig. 1) is significant because it shows phosphate positions in the apatitic lattice are susceptible to ionic substitution. Raman measures of carbonate-to-phosphate (at 959 cm$^{-1}$) ratios can provide valuable insights into the chemical composition of murine or human bones because it varies with bone architecture, age, and mineral crystallinity [8, 14, 15, 16]. The strength of bone is not only dependent on the amount of mineralization, but also on the degree of mineral crystallinity and the optimal distribution of different crystal sizes [17, 18]. Mineral crystallinity was estimated from the inverse of the phosphate 959 cm$^{-1}$ bandwidth at half-maximum [1, 9]. The band ratios corresponding to the same group were pooled together. Data from different groups were compared. The results are quoted in Table 1, as average values ± standard deviation. t-test was used for statistical evaluation.

3. Results and discussion

3.1. Mineral to matrix ratio

MMR provides information on the amount of mineral normalized for the amount of organic matrix within the volume of bone analyzed. The results of the present study indicate that there is no significant difference between male and female specimen. Sex differences doesn’t seem to affect this ratio, as reported elsewhere [5].
3.2. Carbonate to phosphate ratio and crystallinity

Further characterization of the mineral for the animal subjects revealed small and not statistically significant differences in crystallinity and carbonate content between male and female rats (Table 1). These results are in agreement with previous studies [5].

3.3. Amide I

It has been established that amide I band in Raman spectrum is associated with structural properties of collagen. In particular, it is known that it is sensitive to the secondary structure [19, 20, 23] and the ratio between the reducible and non-reducible cross-links of collagen [21, 22, 23]. Non-reducible crosslinks vibrate at 1668 cm\(^{-1}\) approximately while
Figure 4. Deconvoluted bands under amide I envelope. Dotted lines: Experimentally determined, baseline corrected, spectra. Solid black lines: Simulated spectra after fitting of the sub-bands. Solid colored lines: Fitted sub-bands. The wavenumber of the maximum is quoted.

Table 1.
Bone metric parameters and values for the three groups of specimens as determined by Raman spectroscopy. Data are presented as mean ± S.D.

<table>
<thead>
<tr>
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<th>Mineral to matrix ratio</th>
<th>Carbonate to phosphate ratio</th>
<th>Crystallinity (1/FWHM at 959 cm⁻¹)</th>
<th>1668 cm⁻¹/1688 cm⁻¹ band ratio</th>
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<tbody>
<tr>
<td><strong>Male</strong></td>
<td>15.77 ± 2.16</td>
<td>0.191 ± 0.038</td>
<td>0.0564 ± 0.0039</td>
<td>2.09 ± 0.17</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td>16.31 ± 1.10</td>
<td>0.180 ± 0.013</td>
<td>0.0561 ± 0.0026</td>
<td>1.91 ± 0.22</td>
</tr>
</tbody>
</table>

reducible at 1688 cm⁻¹. The relative ratio of these vibrations affects the total area under the amide I band as well as the height of the peak. In our study the amide I band ratio remained statistically similar between sexes of wistar rats.

4. Conclusion

Left tibia and femoral bone from male an female wistar rats were studied with Raman spectroscopy. Raman spectroscopy can be used for evaluating the compositional contributors of bone quality in ex vivo specimens. Several spectra from peristeme were collected. We focused on four regions of interest: the intensity of the primary phosphate band (PO₄³⁻, ν₁) at 959 cm⁻¹, of 1072 cm⁻¹ for the carbonate of the mineral, the matrix bands at 855 cm⁻¹ (proline), 875 cm⁻¹ (hydroxyproline), as well as the 1668 cm⁻¹ and 1685 cm⁻¹ bands under the amide I envelope (1590-1710 cm⁻¹) were measured. The following Raman metrics were calculated: mineral to matrix ratio (MMR), mineral carbonation, mineral crystallinity and [1668 cm⁻¹ / 1685 cm⁻¹] which corresponds to collagen secondary structure. Analysis suggested that no statistically significant difference was noticed between male and female rats.
References