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Mapping Molecular Changes Associated with Avascular Necrosis Using Raman Spectrometry

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Abstract: Introduction: Bone AVN (avascular necrosis) is a pathological condition which results from hypo-vascularity to the bone tissue. Although it is a poorly understood condition, it can be a consequence of either traumatic or non-traumatic factors compromising the bone's precarious blood flow. Bone hypo-vascularity causes the death of bone marrow, osteocytes and ultimate failure of the necrotic part. Due to the lack of resolution and poor sensitivity of existing diagnostic modalities, the aim of this study was to examine necrotic bones, and map the molecular changes using Raman spectrometry. Materials and Methods: Following institutional permission, femoral heads of patients undergoing total hip arthroplasty for AVN secondary to SCD (sickle cell disease) (n = 7) were obtained, and stored at -20 ºC until further processing. Each head was cut in half and scanned with a Raman spectrometer (I- Raman Ex Bwtek system). Scanned areas were correlated with preoperative magnetic resonance scans. Results: The effect of AVN on bone mineralization, crystallinity of minerals, content of carbonate, collagen cross-linking, mineral and collagen fibril orientation were examined. Results suggest a significant difference ($p < 0.05$) in bone quality when comparing diseased bone areas to normal controls. Conclusion: Our results show the efficacy of Raman spectroscopy as a tool to analyse the overall biochemical signature of necrotic bone. It can be a potential screening tool for bone AVN, and provide future predication of bone fragility while differentiating it from other bone pathologies.

Key words: Raman, avascular necrosis, sickle cell.

1. Introduction

Femoral head necrosis is a pathological condition which is characterized by ischemia and death of bone cells (osteocytes). Once osteocytes start to die, it can progress to head collapse and flattening, and results in severe pain and loss of hip joint function. The process of the femoral head AVN (avascular necrosis) is poorly understood, however, the common pathways are known to have both traumatic or none traumatic causes that compromise femoral head precarious circulation, ultimately, leading to secondary osteoarthritis. Femoral head necrosis secondary to SCD (sickle cell disease) is a common musculoskeletal manifestation of the disease pathogeneses [1]. The production of sickle haemoglobin, due to sickled-point mutation in the beta globin gene, can lead to acute and chronic pain or bone tissue ischemia and infarction [2]. Identification of early disease involvement in bone degradation and diagnosis of a vascular necrosis could be valuable given that therapeutic intervention is more effective at early stages [3]. Obtaining the appropriate diagnostic modality to appropriately stage the disease process is considered crucial. Still, despite the availability of multiple imaging modalities for the diagnosis of avascular necrosis, detection of disease progress presents a challenge for the treating physician. This is can be attributed to the lack of resolution and sensitivity of the existing modalities. Given the disappointing outcome of therapy after bone collapse,

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clinicians advocate for early detection of the disease pathology before the onset of structure failure [4]. In this given scenario, the reported work aims to explore the use of vibrational spectrometry as a potential tool for early diagnosis of femoral head AVN induced by SCD.

Raman spectrometry is a powerful optical analytic method which can provide detailed and specific information at a molecular level based on inelastic scattering of light by vibrating molecules. The concept of using vibrational spectroscopic technology as adjunct diagnostic tool in medicine dates back over half century [5]. The aim of this study was to examine necrotic bone and map the molecular changes using Raman spectrometry.

2. Material and Methods

This project has been reviewed and approved by the medical ethical approval committee/college of medicine at Sultan Qaboos University (REF. No. SQU-EC1039114). Femoral heads of SCD patients undergoing total hip arthroplasty (THA) due to advanced AVN (10 heads from 7 patients) were obtained, and stored under aseptic conditions at -20 ºC until further processing. The average age was 25 (22-32 years), and all used femoral heads can be categorized as stage 5 or 6 in the Steinberg classification of femoral head necrosis [6] (Figs. 1a and 1b).

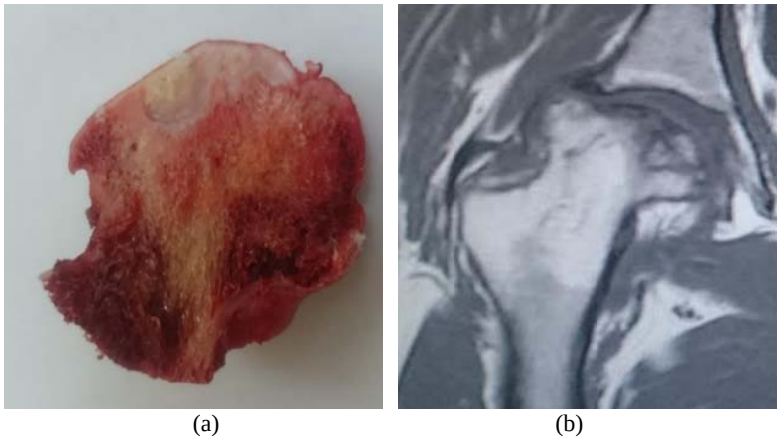
Mapping of the necrotic areas was done using gross appearance and was correlated with magnetic resonance scanning. Raman analysis was done using a Raman spectrometer (I-Raman Ex Bwtek system), excitation was by diode laser operating at 1,064 nm. Spectra were recorded at 400-2,000 cm^{-1}. Each sample was cut into 2 halves and multiple spectra were collected from both necrotic and normal healthy bone areas.

2.1 Spectral Analysis

Collected spectra were baseline corrected using BWSpec software and averaged to improve signal-to-noise ratios. BWSpec software allows manual determination of peak intensity. Bone is a rigid organ comprised of living bone cells which are embedded in a mineralized organic matrix. Thus the effects of avascular necrosis on bone mineralization, crystallinity of minerals, content of carbonate, collagen cross-linking, mineral and collagen fibril orientation were examined. Statistical assessment of Raman parameters of necrotic bone compared to the control was performed to illustrate the changes in chemical composition and internal structure. All statistical analysis was performed with significance set at p value < 0.05.

3. Results

Normal human bone spectra along with the band



(a) (b)

Fig. 1 (a): ex-vivo femoral head which was cut on half; (b): femoral head with advance necrotic changes.

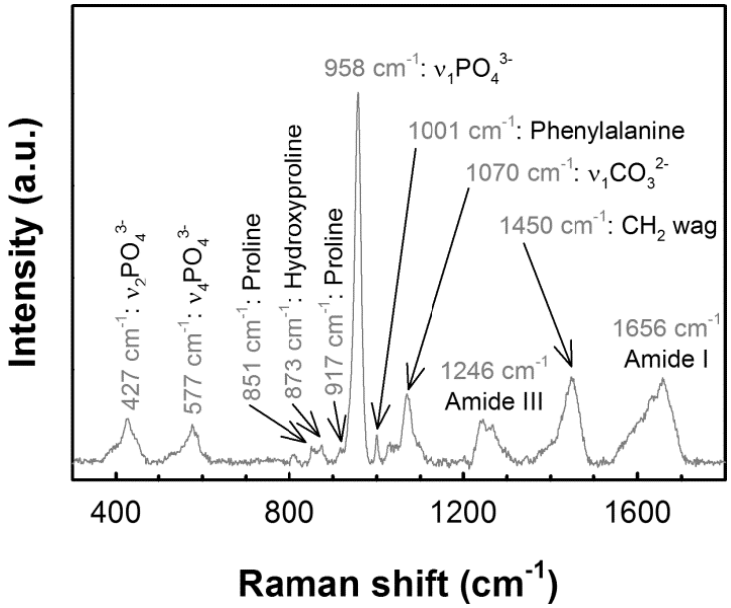


Fig. 2 Normal bone Raman spectra where phosphate peak can be shown at 958 cm^{-1}, amide at 1656^{-1}.

assignment [7] are presented in Fig. 2. The assigned bands represent both bone minerals and the collagen matrix.

Comparing the amount of bone minerals to the organic matrix as a mineral: matrix ratio gives a representation of the bone volume content. In comparison to the control, necrotic bone shows significant change in the mineral to matrix ratio. The ratio was calculated comparing phosphate and carbonate Raman band intensities at 958 and 1,070 cm^{-1} respectively, to the intensity of amide I band at 1,656 cm^{-1}. Hypo-mineralization was observed in necrotic segments, indicated by significant decrease of mineral to matrix ratio ($p < 0.05$).

The carbonate to phosphate ratio provides information on substitution of phosphate by carbonate in the bone mineral crystals. The ratio was calculated comparing the carbonate band at 1,070 cm^{-1} to the phosphate band at 958 cm^{-1}. The carbonate-to-phosphate ratio was observed to significantly increase in necrotic areas compared to the control ($p < 0.05$).

Forty percent of human bone is characterized by organic component; mainly collagen. Collagen represents fibril scaffolds that provide tensile strength and viscoelasticity to the bone. The position of the amide I band was also studied. A significant shift from 1,660 cm^{-1} to 1,684 cm^{-1} in the control compared to the necrotic bone ($p < 0.05$) was observed [8].

4. Discussion

Bone quality is a term which is used to describe the composition and architectural properties of bone tissue that together can determine bone material properties and ability to perform mechanical function [9]. Disturbance of the bone tissue vascularity can result in state of low oxygen and nutrition that interrupts physiological bone remodelling process and bone quality. Hypo-vascularity can inhibit osteoblast proliferation, alkaline phosphatase activity and osteogenesis, resulting in bone resorption. Visualization of the bone composition and structure at a microscopic scale using tools such as Raman spectrometry can provide an insight into the bone's pathological status. In this study systemic examination of necrotic bone samples using Raman spectroscopy was performed. The Raman spectrum represents a molecular fingerprint of bone ultra-structures which can predict the bone quality and potentially help in its treatment.

Bone mechanical strength can be characterized by the mineral to matrix ratio which is rated as one of the

main compositional indicators of bone. The mineral to matrix ratio is here represented by the intensity of the mineral signal (phosphate at 958 cm^{-1} or carbonate at 1,070 cm^{-1}) relative to that for collagen (amide I at 1,656 cm^{-1}). Imbalance in these ratios will have negative effect on the bone mechanical properties resulting in fragility and easy fracture. The study results have shown significant decrease in the phosphate to amide I ratio in the necrotic samples which indicates defective mineralization due to the disease pathology (Fig. 4a) [10]. The study results are analogous to what can happen in osteoporosis where the hormonal levels changes results in abnormal mineralization and decrease in the mineral to matrix ratio.

Changes in the collagen spectra in necrotic bone samples suggest rupture of the cross linking which can result in deformation of fibril scaffolding, and loss of

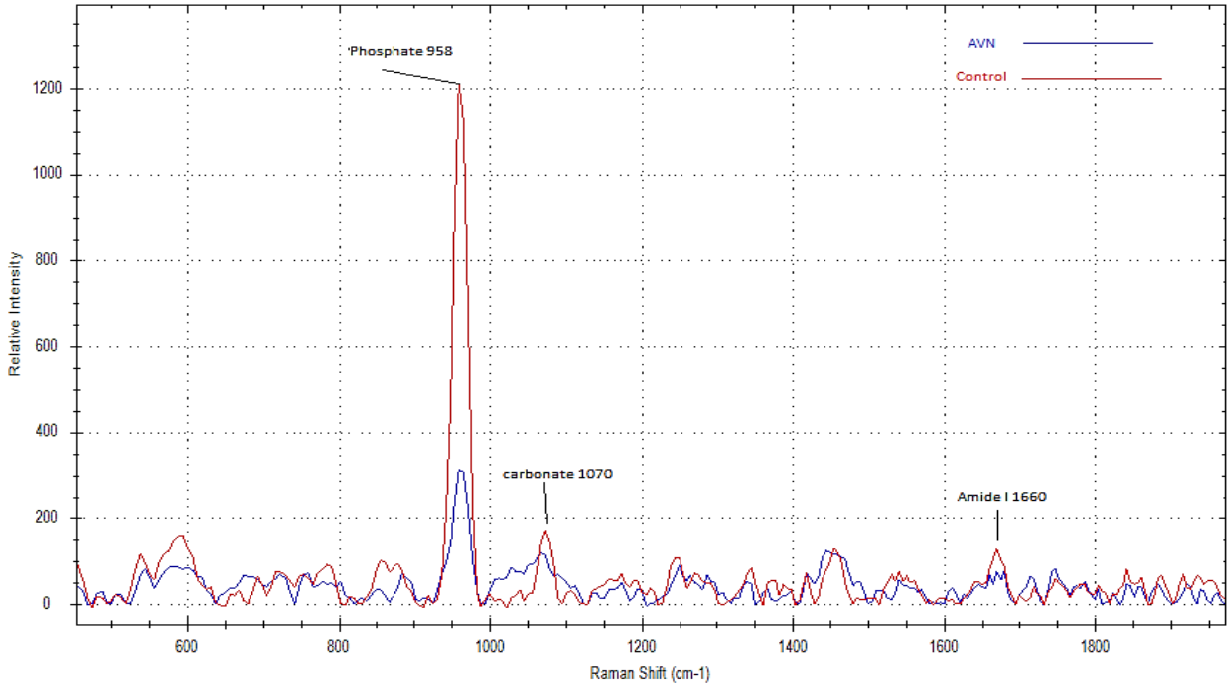


Fig. 3 The significance drops in collected spectra where blue represents necrotic bone compared to red which represents control sample.

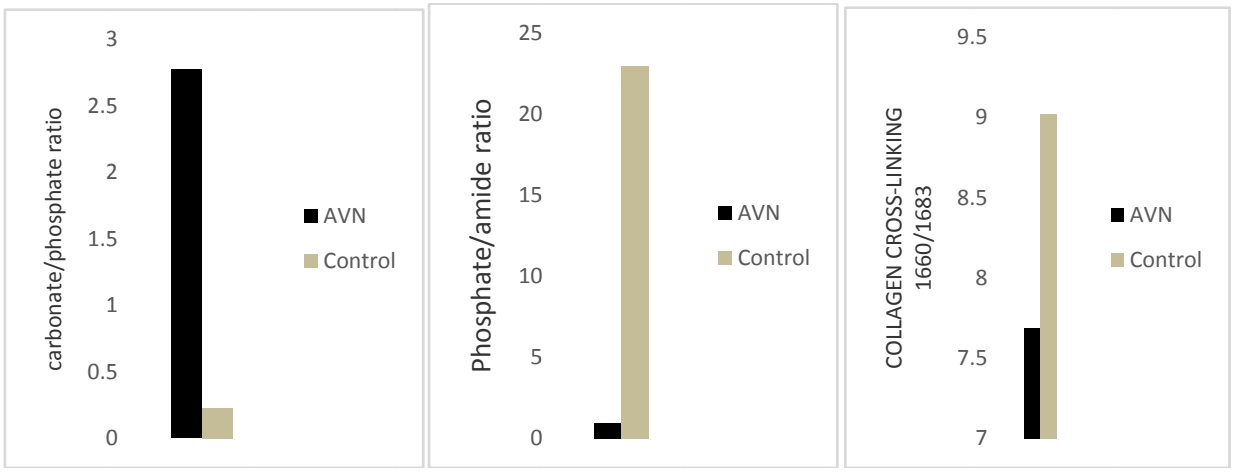


Fig. 4 Bone quality ratio—black graph represents necrotic bone (AVN), grey graph represents control bone samples. (a) the carbonate to phosphate ratio, (b) phosphate to amide ratio, (c) collagen cross-linking ratio.

bone tensile strength. In this study, the collagen cross-linking ratio was significantly higher in the avascular necrotic compare to control (Fig. 4c). The cross linking ratio was represented by amide I band centred 1,660 cm^{-1} relative to amide I band centred at 1,684 cm^{-1}. Bone strength is highly related to the collagen cross-linking. Therefore, altered cross linking will result in bone fragility and decrease in required energy for micro-fracture formation [11, 12]. The change of the collagen quality can be explained by the markedly altered gene expression profile observed in avascular femoral heads [13]. The carbonate-to-phosphate ratio can provide valuable insight to the bone mineral composition. It is estimated as the integrated area of the carbonate peak divided by the phosphate peak and corresponds to the extent of carbonate substitution for phosphate in the bone mineral crystals [7]. It is inversely related to bone remodelling (the ratio increases with the decreased remodeling) [14]. The results show there was an increase on the carbonate-to-phosphate ratio in the necrotic areas compared to the control (Fig. 4b). This is due to the alteration in bone remodelling process which transforms the bone into more brittle tissue that is susceptible to fragility fracture.

5. Conclusions

The present study into bone quality changes caused by femoral head necrosis as a result of sickle cell hemoglobinopathy, advocates for Raman spectrometry as a potential tool for detecting bone pathological processes. This may allow early therapeutic intervention, preventing further bone destruction or loss and fracture.

Limitations

The study results represent bone quality changes in advanced stage of the disease. Future work in samples with early stage disease may give similar changes but with different ratios.

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