ORIGINAL ARTICLE

A CARBON-13 NMR SPECTROSCOPY STUDY ON THE AMINO ACIDS COMPOSITION OF THE MANDIBLE: COMPARISON WITH THE ULNA AND CALVARIA BONES

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ABSTRACT

Bone is a remarkable connective tissue that possesses the quality to undergo the process of remodeling to maintain its mass and structure depending on mechanical loading, however not all bones are subjected to mechanical loading yet they don't show disuse osteoporosis. In oral biology, tooth removal leads to alveolar bone resorption due to reduced mechanical load (like limb bone), however basal bone does not resorb so quickly on disuse despite the fact that there is less mechanical load on it (like calvaria). So in this respect, mandible display features of both the limb bone and calvaria. Therefore the aim was to compare the amino acids composition of mandible with ulna and calvaria and the objective was to compare the bone samples with collagen standard. The commercial collagen type I from the rat tail tendon (sigma Aldrich, UK) was used as a reference to be compared with the bone samples. These samples were subjected to carbon-13 NMR spectroscopy. Results showed that the carbon-13 spectra of ulna and calvaria were almost similar, while mandibular spectra showed the most distinct results. Carbon-13 NMR study showed increased proline content in calvaria then ulna. However mandible showed greater hydroxyproline content and lower glycine content than other spectra. Furthermore, an important finding was the presence of additional amino acids particularly aspartate, leucine and isoleucine in mandible, and glutamate, phenylalanine and methionine in other bone spectra, that might suggests non-collagenous proteins in bone. Additional work is required using new techniques in the NMR that can separate collagenous proteins from the non-collagenous proteins to further explore the complex dynamics of bone.

Key Words: *Amino acids, Carbon-13 NMR, Mandible, Ulna, Calvaria.*

INTRODUCTION

Bone is a specialized dense connective tissue that plays a vital role as a load bearing organ present in the body.[1] Bone contains two types of tissues, 80% is cortical and 20% is trabecular and is made up of bone cells and extracellular matter. Bone cells comprises 2% by weight, whereas the extracellular matter is composed of inorganic and organic matrix, and water.[2]

The inorganic part (65%) is usually the calcium carbonate apatite of type B.[3,4] The organic matrix (25%) is predominantly collagen (mostly type I) which makes up 90% of the organic matrix with remaining 10% formed by non-collagenous proteins. The remaining extracellular matter is water (10%), which is mostly bound to collagen.[5] The interaction of the inorganic and organic parts of the bone defines the mechanical properties of the bone.[3]

It is very important to monitor the health status of the bone owing to the fact that bone is subjected to several complex alterations during the period of lifetime of an individual, that can be both natural and pathological.[6] Apart from obvious strength, one of the most essential property of the bone is its capability to undergo the process of remodelling according to the functional demands placed on it. Same bones rely on mechanical stimuli to maintain its mass and structure[7] and this mechanical loading have a major influence on the bone architecture.[8] However different bones respond differently to the mechanical stimuli. Study by Rawlinson et al in 1995 suggested that there is a possibility that slight variation might exist in the com-

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Received for Publication: March 31, 2014
Accepted: May 14, 2014

position between bones from different sites because of the fact that different bones can withstand different mechanical loads.[9]

In terms of dental tissues, alveolar bone loss can occur due to reasons such as trauma, developmental problems, untreatable endodontic problems and severe periodontal disease[10] and is associated with a reduction in the apico-coronal and bucco-lingual dimensions of the alveolar ridge because the amount of force on the bone is decreased following tooth removal leading to disuse osteoporosis.[11,12] However the basal bone of the mandible is retained despite the tooth loss. This variation within the mandibular bone indicates regulation of bone attributes on a very local level. In the appendicular skeleton absence of the mechanical loading will result in progressive bone resorption called disuse osteoporosis whereas the cranial skeleton experiences very low mechanical load but still retain their structural integrity. So in this respect, the mandible could be considered to be a combination of both the axial skeleton and the cranial skeleton in relation to its requirement for mechanical loading. Study by Kingsmill et al. in 2013 compared mandible with the ulna and calvaria and revealed that genes linked to pathways found in both the mandible and the ulna. Further, the mandible and calvaria which does not respond to mechanical stimuli also share similar pathways[13] which differs from those in the ulna/mandible subset. This study used gene expression to compare the bones but no study have been done so far to study in depth the compositional differences in terms of amounts of amino acids between bones.

Several factors determine the overall quality of the bone such as bone chemical composition, structure, micro-architecture and mineral density. These can be investigated by several techniques but are either non-comprehensive or invasive.[6] The bone morphological diversity and considerable sensitivity of the bone samples to chemical and physical effects causes extreme experimental challenges. As a result wet chemical methods are considered too invasive, particularly when inorganic or organic part of the bone have to be isolated.[9] Furthermore collagen and amino acids have been studied by X-ray diffraction and electron microscopic studies but these methods provides only a static view of the molecule.[6] The high-resolution solid-state NMR enables us to look specifically at the selected magnetic nuclei in the bone without any chemical pre-treatment, therefore avoiding any intervention into the bone structure.[6] Considering the hypothesis that mandible possesses features of both the limb bone and calvaria, ^{13}C high resolution solid-state NMR has been used to compare these bones in terms of amino acids and analyse these bones at the atomic level.

METHODOLOGY

Collagen sample (Fig 1) was purchased from Sigma Aldrich UK Catg # 100MG (Lot # 120M7011V). The collagen was type I from rat tail tendon and was kept in the refrigerator at 10ºC.

Collection of Bone Samples

Mandible, ulna and calvaria bones were taken from rats weighing 200 grams. All the muscles, tendons, ligaments, soft tissues and teeth (in mandible) were cleaned off as much as possible. Bones were scrapped to remove any loose connective tissue attached. Calvaria bone was cut in such a way that there was no suture in the sample. The long bones were prepared by cutting the epiphysis off and the marrow removed since there is cartilage at the ends which in not required for this experiment. Mandibular bone was cut carefully to remove the teeth and any ligaments present. The samples were then snap frozen in liquid nitrogen and stored frozen at -70ºC.

Preparation of Bone Samples

Mortar and pestle were pre-cooled in liquid nitrogen. Bone samples were placed as a group in the mortar and pestle and the liquid nitrogen was poured on them to make them brittle and finally ground down into powder form (Fig 2). Separate pestles and mortars were used for each group. This was to prevent cross contamination of samples. The powder bone samples were then transferred to pre-cooled glass tubes and stored again at -70ºC. Prior to the experiments the samples were brought to room temperature.

The NMR experiments were performed at a field of 14.1 Tesla (T) in a Bruker Spectrometer (Fig 3) operating at a resonance frequency of 600 MHz, using the 4 mm rotor. ^{13}C NMR was run with a resonance frequency of 150.9 MHz. ^{13}C Cross-polarization from protons were done at a spinning rate of 12000 Hz or 12 KHz.

RESULTS

The results of 13C (1H) CP-MAS NMR on collagen are presented first, followed by the 13C (1H) CP-MAS NMR performed on mandibular, ulnar and calvaria bones. Comparison of all the ^{13}C NMR spectra of the bones and collagen is then presented with greater focus on the aliphatic amino acids for analysing any differences present. The results of the comparison between the four samples are summarized in Table 1 below:

Fig 1: Collagen sample

Fig 2: Powdered bone sample after grinding

Fig 3: NMR Spectrometer

Fig 4: Rotor for packing the specimen

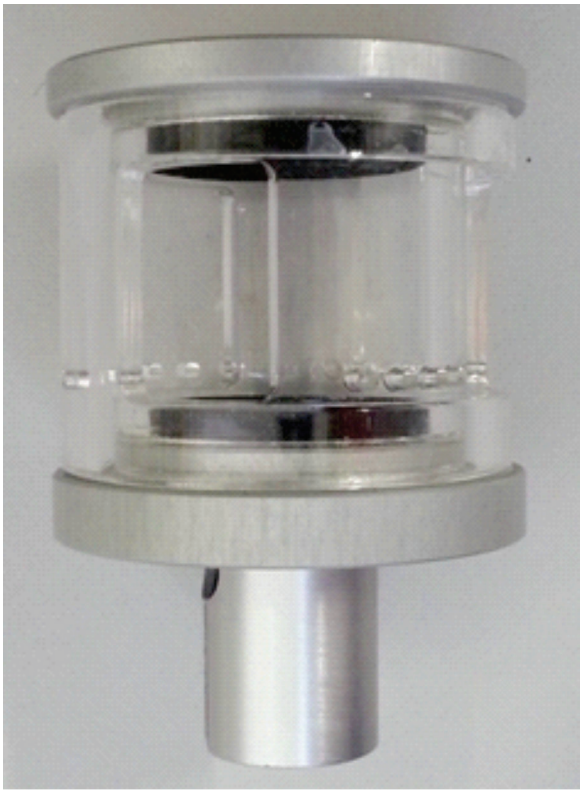

Fig 5: Part of the transfer tube which receive the rotor

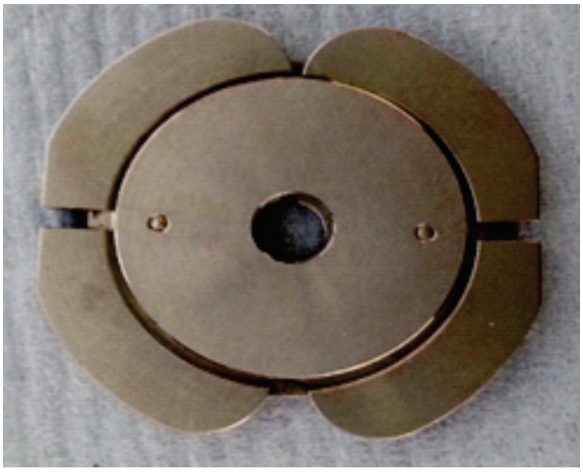

Fig 6: Tools for opening the caps of the rotor

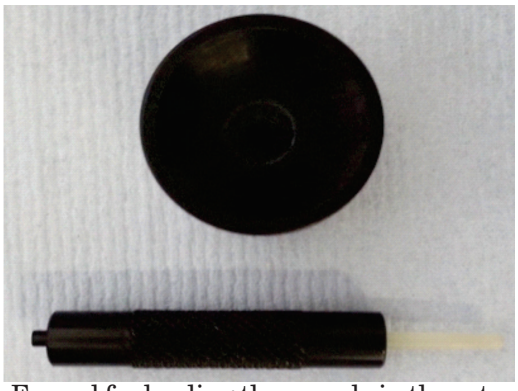

Fig 7: Funnel for loading the sample in the rotor along with the packing tool

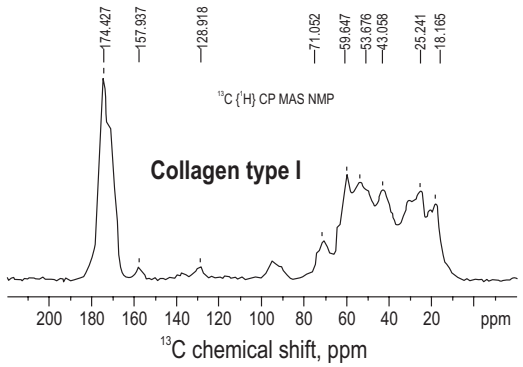

Fig 8: ^{13}C (^{1}H) CP-MAS NMR at 12 kHz spinning speed

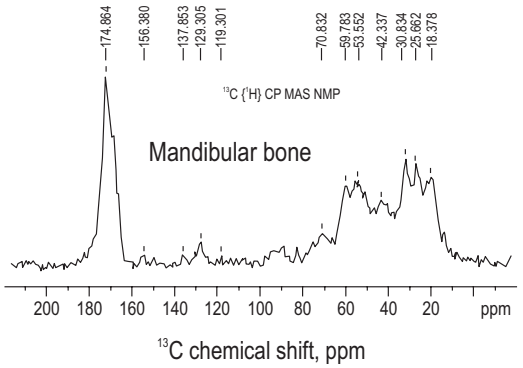

Fig 9: ^{13}C (^{1}H) CP-MAS NMR spectrum of Mandibular Bone

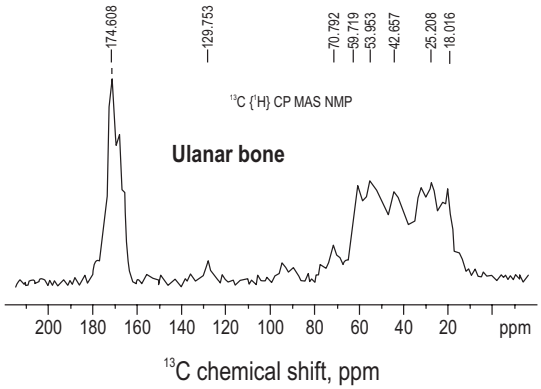

Fig 10: ^{13}C (^{1}H) CP-MAS NMR spectrum of Ulnar Bone

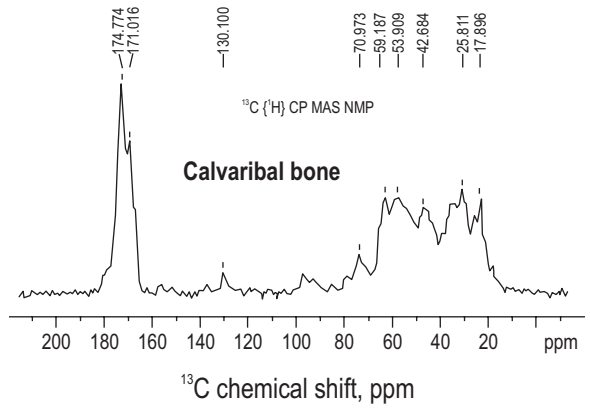

Fig 11: ^{13}C (^{1}H) CP-MAS NMR spectrum of Calvarial Bone

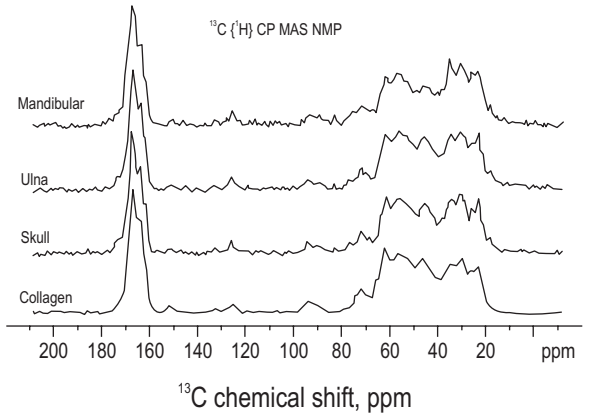

Fig 12: Comparison of ^{13}C (^{1}H) CP-MAS NMR spectra of bones amongst each other and with the ^{13}C (^{1}H) CP-MAS NMR spectrum of collagen

Fig. 12 shows ^{13}C (^{1}H) CP-MAS NMR spectral comparison. The three bones spectra are compared with that of the collagen. Little but considerable differences are seen amongst the peak positions of the aliphatic amino acids in the different bones and collagen. The peak position at about 70 ppm is almost identical in all the spectra with the mandibular spectra showing a little intense peak at this position (assigned to Hydroxyproline Cγ). The peak positions 59 ppm shows greatest intensity in collagen spectra while the mandible showing the least intense peak (assigned to Proline Cα and Hydroxyproline Cα, Cδ) . The peak positions at 53 ppm (assigned to Phenylalanine Cα and methionine Cα) and 42 ppm (assigned to Glycine Cα) appears highest for ulna followed by calvaria and collagen spectra.

The most significant peak positions in mandible spectra appears at 25 ppm (assigned to Proline Cγ), 30 ppm (assigned to Proline Cβ), 37 ppm (assigned to Hydroxyproline C_{β} and Aspartate C_{β}) and 40 ppm (assigned to leucine Cβ, isoleucine Cβ and phenylalanine Cβ) as compare to other spectra.

TABLE 1: THE EXPERIMENTAL ^{13}C CHEMICAL SHIFT IN PPM OBSERVED IN DIFFERENT SAMPLES FROM THE ^{13}C (^{1}H) CP-MAS NMR SPECTRA IN FIGURE 4.5. THIS TABLE IS BASED ON THE LITERATURE SOURCES [13] AND DISCUSSED IN THE TEXT

Sample/Amino Acids	Mandible	Ulna	Calvaria	Collagen
Ala C_β	18.37	18.01	17.89	18.16
Pro C_γ	25.66	25.29	25.84	25.24
Pro C_β, Glu C_β	30.83	30.70	30.80	30.85
Hyp C_β, Asp C_β	37.50	-*	-*	-*
Leu C_β, Ile C_β, Phe C_β	39.80	-*	-*	-*
Gly C_α	42.33	42.65	42.68	43.05
Phe C_α, Met C_α	53.55	53.95	53.90	53.67
Pro C_α, Hyp C_α, C_δ	59.78	59.71	59.18	59.64
Hyp C_γ	70.83	70.79	70.97	71.05
Aromatic region	129.30	129.75	130.10	128.91
C=O	174.86	174.60	174.77	174.42

* severe overlapping of a few peaks at this position made it impossible to accurately identify the position

Mandible spectra also shows most numbers of peaks in the aromatic region (100-160 ppm) as compared to other spectra showing single peak in this region. However the main peak in this region for all four spectra appears at around 129 ppm with calvaria (130 ppm) and collagen (128 ppm) spectra showing one ppm drift.

Peak positions from the carbonyl region (160-200 ppm) appears almost similar for every spectra at about 174 ppm, with calvaria showing an additional peak position at 171 ppm.

DISCUSSION

The solid-state carbon-13 NMR study on the bone samples gave peak positions for the several amino acids present in the bone matrix. The spectra obtained were then compared and evaluated among each other and also with the carbon-13 NMR study on samples of collagen as seen in Fig 12. The CO_3^{2-} signals from the carbonate apatite cannot be visualized due to extremely low content of carbonate in bone apatite type B estimated to be about 5-8% by weight.[2,15-17] Therefore the ^{13}C of carbonate which should come at around 168-170 ppm was totally obscured by the carbonyl signal of the organic matrix at the same position.[6] Assignments of the peaks obtained from the several carbons of the aliphatic amino acids residues were made based on ^{13}C chemical shifts for amino acids, peptides and proteins done in the literature.[6,14,17] The peak positions for the amino acids from the aliphatic region, aromatic region and the carbonyl region in all the four samples are shown in Table 1.

Evaluation of the peak positions and the spectrum, revealed small but considerable differences. From the above mentioned results, ulnar and calvarial spectra were more or less similar except that proline C_β was much more in ulna than in calvaria, whereas Proline C_γ was more in calvaria than in ulna. This can be attributed to the fact that proline C_γ provides more stability to the triple helix as compared to proline C_α, C_δ C_β because proline C_γ gives more flexibility which makes pyrrolidone rings to cause a regular twisting of the polypeptide chains more easily.[18]

Interestingly the spectrum of the mandible was relatively different from the other bones spectra. Glycine content in the mandible was lower than the ulna and the calvaria spectra which might represent the alveolar part of the mandible as it is much more vulnerable to disuse osteoporosis and this lower glycine content may provide the required destability to the collagen structure as higher glycine content is associated with greater stability because it can accommodate within the interior of the triple helix due to its small size.[19] On the other hand higher proline content in the mandible and the calvaria than ulna might represent the basal part of the mandible as it is much less susceptible to the disuse osteoporosis. This is due to the fact the proline in the triple helix provides higher stability to the collagen structure. In addition, the presence of hydroxyproline in greater amount in the mandible further supports this thought that this may represent the basal part of the mandible as hydroxyproline in the collagen provides greater stability than proline in the same position. Both proline and hydroxyproline stabilize the polyproline II extended conformation of each chain in the triple helix due to the stereo chemical restrictions of the imino acid

rings. However hydroxyproline was known to confer a greater stability than proline in the Y position, and this stabilization was shown to be stereospecific and dependent on being in the Y position. The fact that the hydroxyl group of Hyp could not directly hydrogen bond to the carbonyl groups within the same molecule led to the proposal that its effect was mediated through bridging water molecules. It plays a pivotal role in creating the ordered water shell around the triple-helix. Hyp hydroxyl groups linked by water molecules to a Gly C=O within the same chain, and to the Hyp C=O of the adjacent chain, are sufficient to satisfy all hydrogen bonding potential in the chains. Therefore a high Hyp content in the Y position of certain collagen types may thus increase stability through this extensive water network.[20]

Besides the presence of collagenous amino acids, other amino acids were also detected in the bones spectra, especially the mandibular spectrum in which aspartate, leucine and isoleucine were found exclusively than other bone spectra, also glutamate, phenylalanine and methionine were detected in the ulna and calvaria spectra, but they were also present in the mandible especially glutamate. These amino acids might represent non-collagenous part of the bone's organic matrix. Considering our aims to compare the mandibular bone with the ulna and calvaria, the amino acids composition of the mandible was relatively different from the ulna and calvaria and that difference can be taken into consideration, but in order to improve our understanding regarding the more precise differences in amino acids between these bones, more sensitive techniques are needed in which we can separate collagenous part from the non-collagenous part, that may give some additional important clues about the differences between these bones.

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