ORIGINAL ARTICLE

THE STUDY OF DEMINERALIZED AND REMINERALIZED ENAMEL & HYDROXYAPATITE USING NUCLEAR MAGNETIC RESONANCE (NMR)

[1]ADIL DURRANI
[2]MUHAMMAD ANWAAR ALAM
[3]MUSTAFA QADEER
[4]SAIRA ATIF

ABSTRACT

The study was conducted to observe if there is any difference in the chemical structure of demineralized and remineralized enamel and hydroxyapatite using solid state 31P MAS NMR techniques. Chemical structure and composition of enamel and hydroxyapatite powder were analyzed using different acids i.e. hydrochloric acid, citric acid and acetic acid, of different molarities, followed by treatment in remineralizing solution for a time period of 1, 3, 6 and in some cases 7 days, using NMR. We expected to change current concepts of remineralizing and demineralizing processes of enamel and hydroxyapatite.

Synthetic hydroxyapatite provided by Plasma Biotal was used for the experiments, while extracted teeth provided by tissue culture labs were used for the enamel. The enamel crowns were converted into powder using Gyro Mill. All the measurements for NMR were done in Bruker NMR spectrometer which had a magnetic field strength of 600MHz or 14.1 Tesla. The nucleus used for the experiments was Phosphorous-31. Changes were observed in the peak positions of HAP as well as enamel powder samples. Remineralized samples (both enamel and HAP) also showed increase in mass which may be due to precipitation of hydroxyapatite on consuming the ions, used from the remineralizing solution.

This study provides a better insight into the remineralization and demineralization of enamel and HAP and the changes that take place in the chemical structure after and during the processes. The work also demonstrates that NMR is a very powerful and modern technique which can be used to detect structural changes in different complicated materials.

Key Words: *Hydroxyapatite, remineralization, demineralization, NMR.*

INTRODUCTION

Erosion and caries are two of the most prevalent diseases effecting population around the world. These diseases severely affect enamel of the teeth which is highly mineralized and hardest tissue of human body. The enamel consists of carbonated calcium hydroxyapatite crystals, the structure of which makes enamel surface resistant to acid attack.[1] The demineralization of enamel causes dissolution of HAP crystals, which occurs as a result of intake of acidic food, beverages and bad oral hygiene ultimately leading to cavitation and caries. Most of the process of demineralization takes place at or around a pH of 5 (Below 5.5) also known as the "critical pH".[2] Remineralization is a major process that helps repair the enamel surface and crystals after such an attack. Partial reconstitution of destroyed enamel tissue has been known to occur both in natural and artificial in vitro conditions.[3] Oral cavity has the capability to remineralize the demineralized enamel through the supply of calcium and phosphate ions in saliva to the teeth. The processes of remineralization and demineralization have very important roles in our life and environment.[4]

Different studies have been conducted on the structure and chemical composition of hydroxyapatite and enamel by using NMR. Over the period this technique has played a pivotal role in discovering the mechanism and chemical interactions at molecular level.

[1] Adil Durrani, BDS, MSc, Assistant Professor, Department of Oral Biology, Frontier Medical College, Abbottabad, Pakistan
Cell: +92-302-4721317 Email: adil_durrani@hotmail.com
[2] Muhammad Anwaar Alam, BDS, MSc, Assistant Professor, Department of Oral Biology, Multan Medical and Dental College, Southern Bypass, Jahangirabad, Multan, Pakistan
Cell: +92-0321-4277377 Email: dranwaaralam@hotmail.com
[3] Mustafa Qadeer, BDS, MSc, Assistant Professor, Department of Oral Biology, Institute of Dentistry, CMH, Lahore. Medical College, Abdur Rehman Road, Lahore Cantt, Pakistan
Cell: +92-300-4465123. Email: mustafaqadeer@hotmail.com
[4] **(Corresponding Author)** Saira Atif, BSc, BDS, MPhil, HOD/ Assistant Professor, Department of Oral Biology, Institute of Dentistry, CMH, Lahore Medical College, Abdur Rehman Road, Lahore Cantt, Pakistan Cell: +92-307-8875574
Email: amin_saira@hotmail.com

Received for Publication: August 6, 2015
Revised: January 17, 2016
Approved: January 22, 2016

It can calculate parameters of chemical shifts.[5,6] It also gives information on the local bonding environment around a specific atom and can be calculated for extended period of time. NMR with advancements over the time can now use pseudo wave function to gain information of large complex structures such as hydroxyapatites.[7] We expected to change the current views and concepts about remineraliztion and demineralization processes of enamel and hydroxyapatite through this study.

METHODOLOGY

In this study, two sets of materials were used. The first set was of stoichiometric HAP "Hydroxylapatite" by Plasma Bio Tal Limited (Sintering Grade, Batch P 218 R Spray dried). The HAP powder was placed in glass jars of 500ml volume and placed on top of the magnetic stirrers to continue with either demineralization or remineralization. For demineralization, for each 5 g of HAP a 250 ml of demineralization solution was used and for remineralization of 1 g of HAP or enamel, 125ml of solution was used.

The second material used for study of dissolution was enamel from carious free crown portions of extracted teeth (permanent incisors, canine and molars) which were stored in 70 percent ethanol. The enamel and dentin were not separated because of unavailability of IR spectroscopy. Crowns were converted into fine powder by using gyro mil. The dissolution study was done using a number of acids namely acetic acid (CH_3COOH), hydrochloric acid (HCl) and citric acid ($C_6H_8O_7$.) Acetic acid of different molarity ranging from 0.1 to 1 M was used. The solutions were prepared using acetic acid and bringing the pH of the final solution up to 4 by adding 1 M NaOH to maintain the same fixed pH values for all acetic acid solutions used. The molarity

TABLE 1: MOLECULAR WEIGHT AND MOLARITY OF THE REMINERALIZING AGENTS

Materials	Molarity Mol/ Litre	Molecular wt. Grams (g)	Amount in Sol. g/ litre	Vol of each solution in ml
$CaCl_2$	2×10^{-3}	147	2.94	100
KH_2PO_4	1.2×10^{-3}	136.04	1.63	100
NaF	0.05×10^{-3}	41.8	2.099	1
NaCl	150×10^{-3}	58.5	8.775	8.775 g. Direct

TABLE 2: COMPARISON OF FULL WIDTH HALF MAXIMUM (FWHM) & PEAK VALUES OF HYDROXYAPATITE IN REACTION WITH DIFFERENT ACIDS AND REMINERALIZATION

Process	Treatment	Days	FWHM, Hz	Peak Position ppm
As-received	No	—	243.8	2.87
Dried at 120°C	No	0.5	262.2	2.87
Dried at 200°C	No	0.5	224.3	2.82
Demineralization	Citric Acid	3	272.4	2.84
Demineralization	HCl	3	270.4	2.86
Demineralization	CH_3COOH			2.92
Remineralization	HCl^+	3/6	272.8	2.87
Remineralization	CH_3COOH^+	3/6	261.8	2.87
Remineralization	Remin	7	260.9	2.86

TABLE 3: COMPARISON OF FULL WIDTH HALF MAXIMUM (FWHM) AND PEAK VALUES OF ENAMEL IN REACTION WITH DIFFERENT ACIDS AND REMINERALIZATION

Process	Treatment	Days	FWHM, Hz	Peak Value, ppm
Untreated	No	—	551.2	3.16
Demineralization	Citric Acid	3	443.4	3.11
Demineralization	Acetic Acid	3	455.3	3.09
Remineralization	Citric $Acid^+$	3/3	603.0	3.36
Remineralization	CH_3COOH^+	3/3	629.6	3.40
Remineralization	Remin	1	580.8	3.17

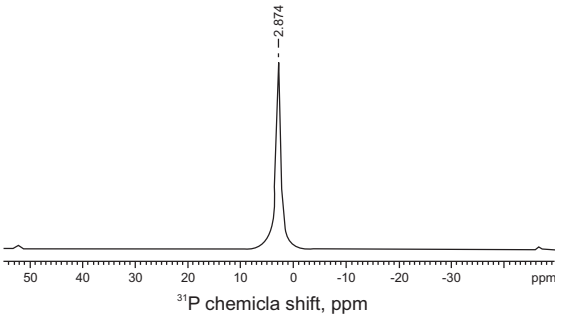

Fig 1: ^{31}P MAS NMR spectrum of HAP showing chemical shift of the main signal in ppm

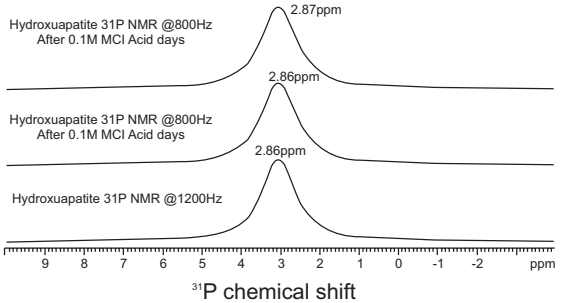

Fig 2: ^{31}P MAS NMR spectra showing ^{31}P Chemical Shift of HAP powder using 0.1 M HCl for period of 3 (middle) and 6 (top) days compared with untreated HAP (bottom)

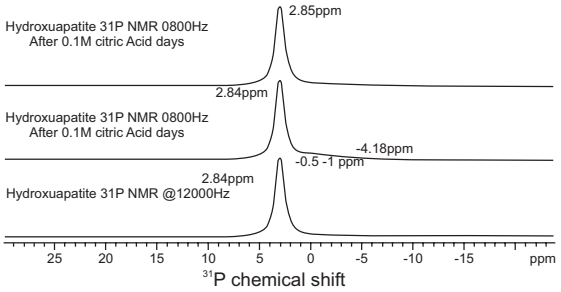

Fig 3: ^{31}P MAS NMR spectra of HAP using 0.1 M citric acid at 3 days (middle) and 6 days (top) compared to untreated HAP (bottom)

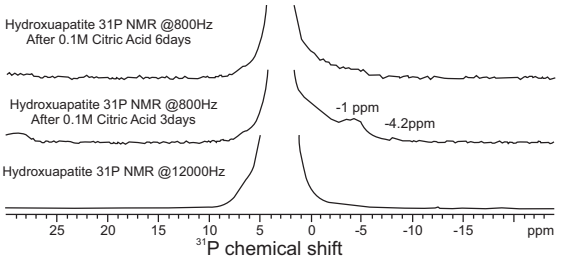

Fig 4: ^{31}P MAS NMR spectra of HAP in citric acid. These are the same spectra as in figure 3 but here are magnified in the foot of the main signal to demonstrate extra peaks as indicated

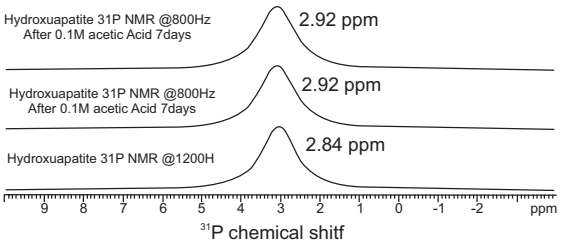

Fig 5: 31P MAS NMR spectra of HAP using 0.1 M acetic acid for a period of 3 days (middle) and 6 days (top) compared to untreated HAP (bottom)

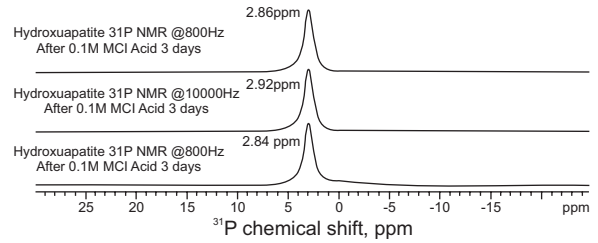

Fig 6: Comparison of 31P MAS NMR spectra of HAP with all 3 Demineralizing Solutions: Hydrochloric acid (top), Acetic acid (middle) and Citric acid (bottom)

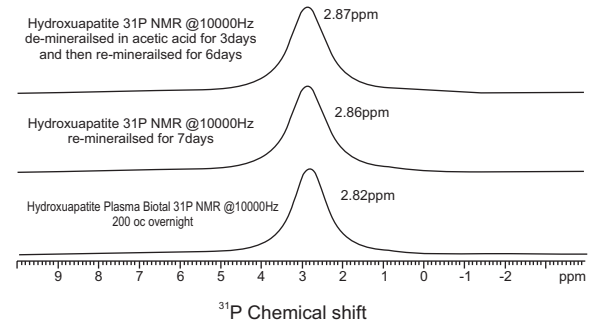

Fig 7: Comparison between HAP dried at 200°C (bottom), remineralized for 7 days (middle) and demineralized in Acetic acid and then remineralized for 6days (top)

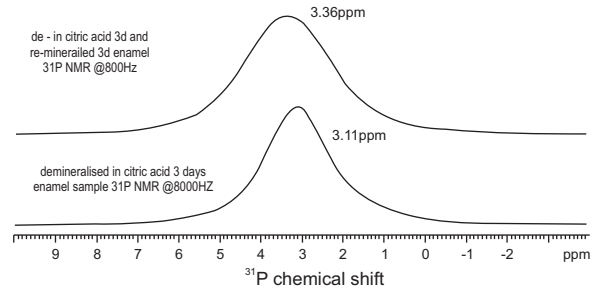

Fig 8: 31P MAS NMR spectra of Enamel demineralized in Citric acid for 3 days (bottom) and then remineralized for 3 days (top)

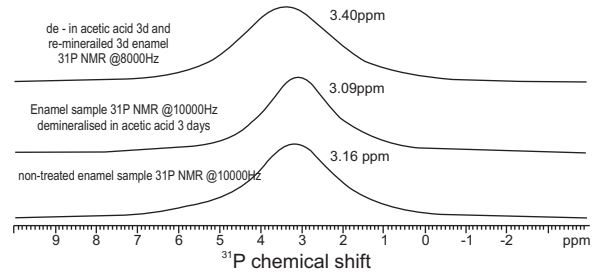

Fig 9: ^{31}P MAS NMR spectra of Normal Enamel (bottom) compared to Demineralized Enamel in Acetic acid for 3 days (middle) and enamel Remineralized for 7 days (top)

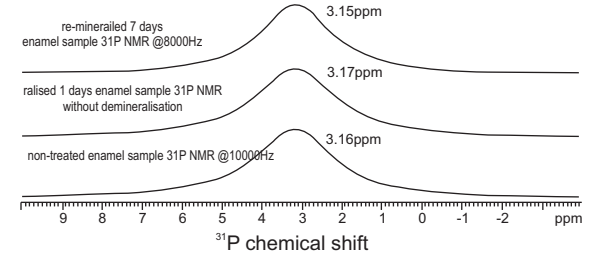

Fig 10: 31P MAS NMR spectra of Normal enamel (bottom) compared to Enamel which has been remineralized for 1 day (middle) and 7 days (top)

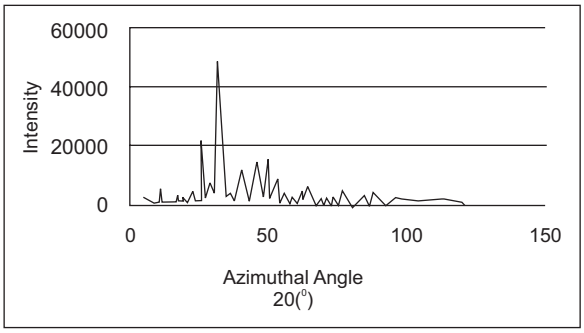

Fig 11: X-ray diffraction pattern of HAP

used for citric acid was finalized at 0.1 M similar to acetic acid. pH was maintained at 2.8. Hydrochloric acid was prepared in different molarities from the 0.1 to 1 M. It was used as a testing agent in this study to check how a strong acid will react with HAP and enamel. It was prepared in the similar manner to the other acids and the recommended molarity set for it was decided at 0.1 M. Higher concentrations simply dissolved Hap powder either immediately (1M) or few min after (0.2M).

The remineralizing solution was made according to the remineralizing solution used in the article by Anderson et al.[8] The main components for the remineralizing solution included calcium chloride, potassium dihydrate phosphate, sodium fluoride and sodium chloride (Table 1). Samples containing 5 g of HAP were placed in remineralizing and demineralizing solution for a time period of 1, 3 and 6 days respectively. After interaction with the solutions they were filtered out using filter paper of 100 micron pore size. These filter papers with the salt were dried incubator overnight. Similarly, enamel powder was placed in demineralizing and remineralizing solutions for the same time period of 1, 3 and 6 days respectively. For each gram of enamel powder 125ml of remineralizing and demineralizing solution was used. While some of the demineralized enamel was again placed in the remineralizing solution to see if there is any further changes in the NMR spectra. All the measurements were done at room temperature using a 4mm Zirconia rotor. The ^{31}P chemical shifts were referenced to 85% H_3PO_4. The signal of the ortho phosphoric acid was assigned to 0 ppm for the reference. The software used for the spectra in the computer was Bruker TopSpin.

RESULTS

In the experiments that we conducted enamel and hydroxyapatites were subjected to demineralization and remineralization using acids and solutions of different concentrations.

DISCUSSION

The FWHM values of all enamel and HAP samples clearly shows the most significant changes seen after remineralization processes in both cases (Table 2 & 3). More significant changes in terms of drift to higher chemical shift values were observed for the enamel samples than for HAP is related to the presence of specific surface layer in enamel which is slightly different from the stoichiometric apatite and is rich with other minor additives from ionic substitutions. The normal HAP spectrum showed the peak at 2.84 ppm (Fig 1) which corresponds to the peak position already seen in literature in studies conducted by W. P. Rothwell et al. in 1979.[9] However, apart from that, in the normal peak there were 2 small peaks detected at the foot of the peak; one at 1 ppm and the other at 5 ppm. This might be due to the presence of protonated and un-protonated hydro phosphate groups found on the surface of hydroxyapatite.[10]

The formation of 2 small peaks was quite intriguing in case of normal HAP and was more evident in case of spectrum of HAP that was demineralized in hydrochloric acid. Further investigated were done by heating HAP sample to 120°C and 200°C. The peak position changed from 2.87 ppm to 2.81 ppm at 200°C. This can be due to the fact that evaporation of water from samples caused the loss of protonated and un-protonated phosphate groups from the surface, leading to decrease in the phosphate signal and resulting in lower peak level. FWHM values recorded also show that the width of 120°C HAP sample was 262.15 Hz which reduced to 224.30 Hz at 200°C. This confirms the findings by Kolmas et al.[11] which stated that the decrease in full width half maximum is directly proportional to the increase in the calcination temperature.

The demineralization process of HAP showed that even though there was not much change in the peak position but there was change in width and broadness of the peaks. The decrease or no change in the peaks, as in the case of hydrochloric acid, shows that there was no change in the chemical structure of hydroxyapatite. There was definite loss in the mass of the samples after demineralization which could be explained by simple process of dissolving of HAP in the acid involved.

The reaction of HAP with citric acid was quite eventful as there was formation of 2 extra peaks in the spectrum apart from the single large peak in the centre. The reason for the appearance of these peaks, in our understanding, could be because the reaction

between citric acid and hydroxyapatite was in a transitory stage, as these peaks almost disappeared when the sample was demineralized for 6 days and were similarly absent in case of enamel samples. Citric acid can form complexes in solutions with different cations due to chelating ability of this molecule. It is possible that this kind of surface species involving phosphate group are formed at the early stage of HAP demineralization. It is not clear why the same did not occur in enamel as we considered enamel surface to be more active than that of HAP.

All the spectra of HAP, subjected to remineralization, showed slight change in peak positions of the phosphorous signal. There was also increase in FMHM of the remineralized peaks and the mass gain after the process (Table 2). This increase can be attributed to the precipitation of HAP in the remineralizing solution which already has calcium and phosphate as its contents. This precipitation caused a slight increase in phosphorous signal leading to increase in peak position and mass. It is possible that on remineralization, a small fraction of other cations present in the remineralizing solution accommodated in the lattice of the precipitated apatite. Some of the cations, e.g. Na^+, would cause change in the chemical shift of phosphorus, shifting it to the more positive value.

In the enamel samples, there were differences seen in peak position as well as FMHM values of the peaks (Table 3) because enamel has a slightly different surface composition as compared to synthetic hydroxyapatite. As this surface layer is the reason for the ionic substitutions that take place in the oral cavity, leading to interaction between hydroxyapatite in the enamel and different ions like fluoride, calcium, zinc, magnesium, phosphate and carbonate etc. These ions can cause shift of the NMR spectrum to higher ^{31}P chemical shift of phosphorus in mineral due to change in the chemical composition of the surface layer. Another reason for the change can be due to difference in the stoichiometry among enamel and synthetic hydroxyapatite. The synthetic hydroxyapatite has a fixed stoichiometric formula. However it is not the same case in enamel. The enamel or the biological apatite has quite a lot of difference in the calcium to phosphorous ratio in its structure leading to a lot of calcium deficient areas. Ca/P ratio seen in stoichiometric HAP is 1.67 and that of enamel is around 1.62. This deficiency leads to increase activity on the surface of enamel leading to more substitution and inevitably changing the chemical composition, causing the chemical shift in the peak of NMR. Also the size of the enamel Nano crystals is much smaller as compared to the hydroxyapatite crystals that we used in this study.

X-ray diffraction pattern of the hydroxyapatite powder shows the relation of intensity and azimuthal angle (2 theta angle). Well defined sharp peaks represent the normal pattern that HAP has and shows the crystalline structure. Sharper peaks mean more crystallinity in the structure of the powder in question (Fig 11). However when we looked at the enamel XRD pattern we found out that the enamel peaks are not symmetrical as well. And it can be a possibility that there could be presence of those 2 minor peaks.

The normal sample of enamel was remineralized in the remineralization solution. They showed a similar increase and decrease in the FWHM character as well as an increase in the width of the peak that was remineralized for 1 day at the value of 580.75 Hz and a lower value of 532.53 Hz for 7 days. This can in a way support the findings in the literature of Tomson et al.[12] that the maximum amount of remineralization is during the first 24 hours and then it tappers down in the next 3 days to become constant at one rate.

The enamel samples were subjected to remineralization in acids shows an increase in the peak size and broadness of the spectrum. The peak is recorded at a very high level of 3.40 ppm which can be owed to the unique surface characteristic of enamel and its ionic substitution and precipitation of HAP in the solution. It was evident from the increase in the sample size as well after the remineralization.

The FWHM values showed a similar pattern with the demineralized enamel sample having a width of only 455.32Hz as compared to the astonishing value of 629.58 Hz.

Similar to acetic acid samples, citric acid reacted samples showed the same behavior in case of enamel. The peak of the demineralized enamel was recorded at a level of 3.11 ppm which has increased to a level of 3.36 ppm after remineralization (Fig 8). The FWHM showed a similar increase from a value of 443.42 Hz in demineralized sample to an unbelievable level of 603.03 Hz after remineralization.

This study provides a better insight into the remineralization and demineralization of enamel and HAP and the changes that take place in the chemical structure after and during the processes. It proves that NMR is a very powerful and modern technique which can be used to detect structural changes in different complicated materials. It can be concluded that indeed

there is a difference between the apatite/mineral precipitated on remineralization and the bulk mineral in oral environment. The difference is mostly in stoichiometry and minor substitutions. Further studies will help to clarify these changes.

REFERENCES

1 Robinson C, Shore RC, Brookes J, Strafford SR, Wood SR, Kirkham J. The chemistry of dental caries. Critical reviews of oral biology and medicine 2000; 11: 481-95.

2 Stephan RM. Changes in Hydrogen-Ion Concentration on Tooth Surfaces and in Carious Lesions. JADA 1940; 27: 718-23.

3 Backer Dirks O. Posteruptive changes in dental enamel. J Dent Res 1966; 45: 503-11.

4 Liu XY, Lim SW. Templating and supersaturation-driven anti-templating: principles of biomineral architecture. J Am Chem Soc 2003; 125: 888-95.

5 Chappell H, Dyer MJ , Groom N, Pickard C, Bristowe P. Probing the surface structure of hydroxyapatite using NMR spectroscopy and first principles calculations. Phys Chem Chem Phys 2008; 10: 600-60.

6 Duer MJ. Introduction to Solid-State NMR Spectroscopy. Blackwell Science, Oxford, 2004.

7 Pickard CJ, Mauri F. Phy Rev B: Condens. Matter Mater. Phys., 2001, 63, 245101.

8 Anderson P, Bollet-Quivogne, FRG, Dowker SEP, Elliott JC. Demineralization in enamel and hydroxyapatite aggregates at increasing ionic. Strengths. Archives of Oral Biology 2004; 49: 199-207.

9 Rothwell WP, Waugh JS, Yesinowskit JP. High-Resolution Variable-Temperature 'P NMR of Solid Calcium Phosphates. Journal of the American Chemical Society 1980; 102: 8.

10 Jarlbring M, Gunneriusson L, Forsling W. Characterisation of the protolytic properties of synthetic carbonate free fluorapatite. J Colloid Interf Sci 2005; 285(1): 206-11.

11 Kolmas K, Slosarczyk A, Wojtowicz A, Kolodziejski W. Estimation of the specific surface area of apatites in human mineralized tissues using 31 p mas NMR. solid state nuclear magnetic resonance 2007; 32: 53-58.

12 Tomson MB, Barone, JP, Nancollas GH. Precise Calcium Phosphate Determination. Atomic Absorption Newsletter 1977; 16: 117-18.

CONTRIBUTION BY AUTHORS

1	**Adil Umar Durrani:**	Contribution to the writing, conception and design, acquisition and interpretation of the data.
2	**Muhammad Anwaar Alam:**	Drafting of the article and data analysis.
3	**Mustafa Qadeer:**	Collection and analysis of the data.
4	**Saira Atif:**	Contribution in drafting and finalizing and proof reading the article. Gave expert research opinion and experience in finalizing the manuscript.