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Spectroscopic analyses reveal radiotherapy-induced variations in elemental composition and crystallite properties of human permanent teeth enamel

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ABSTRACT

Objective: To study the effect of radiation therapy on the structural and elemental composition of permanent teeth enamel *in vitro*.
Methods: Sections from 21 noncarious healthy human teeth were exposed to a cumulative radiation dose of 20–80 Gy. The sections were subjected to electron dispersive spectroscopy (EDS), Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) analysis to study the elemental composition, the ratio of inorganic and organic content, and the mineralization and crystalline properties of the hydroxyapatite crystal structure respectively. All measures were taken on specified areas of enamel surface before and after radiation exposure and compared.
Results: In FTIR and EDS studies, the calcium to phosphorus (Ca/P) and carbonate to phosphate (CO_3^{2-}/PO^{3-}_4) ratios were significantly different ($P < 0.05$) in teeth sections exposed to 80 Gy, indicating the deterioration of inorganic calcium and phosphorous content. The XRD spectrum data showed loss of peaks at seven specific 2θ coordinate areas, flattened peaks and an increase in the crystallite size in the radiation-exposed groups due to mineralization loss and alteration of the hydroxyapatite crystal matrix in the tooth enamel.
Conclusions: Radiotherapy can induce significant variations in the inorganic and organic functional groups constituting the tooth enamel surface; and these variations are dose dependent. The mechanism responsible for delamination and radiation caries needs to be explored by studying the protein lysis pattern, which might be a leading factor causing the enamel degradation and radiation caries.

Abbreviations: HNCs, Head and neck cancers; EDS, electron dispersive spectroscopy; ATR-FTIR, Attenuated total reflectance- Fourier transform infrared spectroscopy; XRD, X-ray diffraction; RT, Radiation therapy; TPS, Treatment planning system; TCP, Tumor control probability; NTCP, Non target tissue complication probability; ORN, Osteoradionecrosis; QOL, Quality of life; FE-SEM, Field emission scanning electron microscopy; XRD, X ray diffraction; PBS, Phosphate buffer saline; LINAC, Linear accelerator; FWHM, The full width at half maximum; JCPDS, Joint Committee on Powder Diffraction Standards; DAE-BRNS, Department of atomic energy- Board of research in nuclear sciences; DST-PURSE, Department of science and technology- Promotion of university research and scientific excellence; CIF, Central instrumentation facility; MIT, Manipal institute of technology.

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1. Introduction

Radiation therapy (RT) plays a major role along with surgery, chemotherapy and other procedures for curative and palliative treatment of head and neck cancers (HNCs) [1,2]. Depending on the type of HNCs, the non-target tissues like bone, teeth, mucous membranes, and salivary glands surrounding the teeth face risk of radiation damage, in spite of careful treatment planning [3]. Teeth are one of the important nontarget oral tissues receiving inadvertent exposure of scattered radiation doses resulting from targeted exposure of tumor sites [4]. During the RT procedure, the treatment planning system (TPS) is used to regulate various parameters like radiation dose, target tissue site logistics and delivery of an optimal radiation dose to the target tumor area so that the maximum tumor control probability (TCP) is achieved, and at the same time, the non-target tissue complication probability (NTCP) can be kept at minimum [5]. Even in the era of treatment by advanced RT modalities, non-target normal tissues are still at risk of radiotherapy-induced side effects like xerostomia, mucositis, osteoradionecrosis (ORN), radiation caries, and teeth attrition [6,7]. In spite of the palliative and prospective care of patients, their quality of life (QOL) is affected due to the side effects of RT on normal tissues [8,9].

Effects of scattered doses of RT on teeth are critical because they contain denser electrons per unit volume, as compared to the soft tissues of the surrounding buccal area. This is responsible for higher dose deposition in the soft tissue-tooth interface region, including muscles and the circulatory system which supplies nutrients to the teeth. This kind of dose concentration makes teeth one of the most vulnerable nontarget tissues to be affected by scattered doses of RT [10].

Therapeutic doses of ionizing radiation can cause changes in the crystalline structure, variations in functional groups, changes in mineralization properties of teeth and at the molecular level, it could also alter the expression of certain proteins [11,12]. Many different material characterization techniques are available to evaluate the structure and chemical composition of teeth samples. Using techniques such as field emission scanning electron microscopy (FE-SEM), microhardness, and surface profilometry, certain structural and morphological variations in enamel and dentin areas due to RT have been established [13–15]. Similar observations were made with respect to the enamel crystal structure and the nanomechanical properties showing a certain degree of deterioration in teeth exposed to increasing doses of RT [16,17].

Simulated RT using *in vitro* conditions can be used to understand the direct effects of RT on teeth. Raman micro-spectroscopy and Fourier transform infra-red (FTIR) spectroscopic studies have shown increased carbonate to phosphate ratios, implying loss of inorganic phosphate content of the hydroxyapatite at a higher rate than the loss of the carbonate content [11]. X-ray diffraction (XRD) analysis has shown differences in the crystallite size and crystallinity of the enamel material [16]. However, there are contradicting results in some studies maintaining that the measure of loss in the carbonate functional group is more pronounced in the hydroxyapatite structure [18]. Therefore, it is believed that many factors may be involved in enamel delamination, loss of surface properties resulting in radiation caries and teeth loss.

In this work, the focus was to study the direct effects of different doses of RT on extracted human teeth using EDS, FTIR spectroscopy and XRD on powdered enamel samples.

2. Methodology

2.1. Sample preparation

Freshly extracted human permanent premolar teeth (n = 21), non-carious, without any attrition/abrasion, were examined and collected from the Yenepoya Dental College after obtaining ethical clearance from the Institutional ethics committee. Patient consent was not required in this study as the patient information was delinked before collecting the samples from clinics. Collected samples were preserved in pH 7.4 phosphate buffered saline (PBS). Hemi sections of the teeth were obtained in the bucco-lingual orientation with a diamond disk and diamond carbide burr attached to an electric micro motor (Fig. 1). Enamel surfaces of Lingual sections (n = 21) were used for EDS analysis before exposure (control) and 12 of them (3 each per dose group) were used for radiation exposure of 20 Gy, 40 Gy, 60 Gy, and 80 Gy (Fig. 1b.). Whereas, for FTIR and XRD studies, enamel parts from buccal sections (Fig. 1a.) were mechanically separated from the dentin and ground using a dental carbide burr with a constant run over of deionized water to prevent burning of the tooth tissue and to prevent major loss of material properties. The slurry containing tooth enamel particles was collected and placed in a hot air oven (60 °C) to dry. The dried enamel powder of teeth was collected, weighed and transferred to labeled Eppendorf tubes. A total of 10 buccal section samples were used with n = 1 as a control and n = 4 for each treatment group.

2.2. Radiation exposure

Radiation exposure was conducted using a 30 × 30 × 30 cm^3 water phantom set up placed on the couch of a medical Linear accelerator (LINAC), Clinac DMX (Varian Medical Systems, Palo Alto, CA); the source to water surface distance was 100 cm. The ionization chamber was set to a 1.5 cm depth at the center of the water phantom and the couch was adjusted in such a way that the beam axis passed through the center of the active volume of the ionization chamber (Fig. 2. The dose per monitor unit was calculated by using the meter reading and other 1 relevant factors. The teeth samples were set on a thin perspex sheet and the sheet was fixed in the water phantom at a depth of 1.5 cm. All teeth samples were at least 1 cm in from the inside of the radiation field (away from the penumbral region) and from the field border. Enamel-dentin buccal sections were irradiated using 6 MV x-rays from the LINAC. Doses

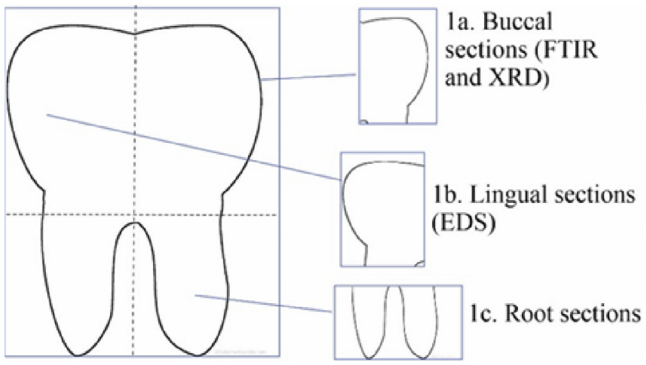

Fig. 1. Sagittal sections of premolar teeth to separate the root from the crown (c.), followed by vertical sectioning in the bucco-lingual orientation to obtain separate buccal (a.) and lingual sections (b.).

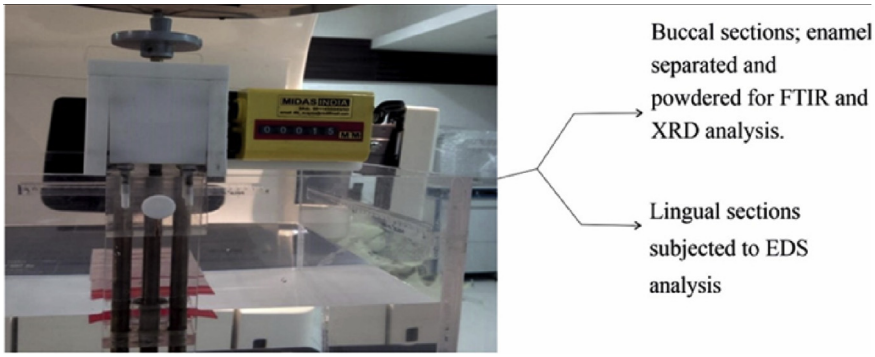

Fig. 2. The radiation exposure procedure with the water tank phantom followed by EDS analysis of the enamel regions of the lingual teeth sections and XRD, FTIR analysis of the powdered enamel of from the buccal teeth sections.

ranging from 20 to 80 Gy were delivered in steps of 20 Gy at a rate of 2 Gy/min.

2.3. FESEM and EDS spectroscopy

The distribution of mineral components and the elemental composition was observed and analyzed using Field emission scanning electron microscopy (FESEM) and Energy dispersive X-ray spectroscopy (EDS). For this, the enamel areas of lingual teeth sections were sputter-coated with gold (Au) and observed using FESEM (Carl Zeiss, Germany). The regions of interest were further amplified to 1000–20,000X and the composition and distribution of elements were analyzed by EDS (Oxford Instruments, England). Other measurements settings included: a direct electron transfer (DET) area of 100 mm^2; window signal A, In-Lens resolution at 10 keV, and a working distance of 12.5 mm. The instrument was calibrated against a Cu standard and operated at 10 kV. The relative abundance of the emitted X rays vs. their energy upon bombarding the sampled volume by the SEM's electron beam was detected by an EDS X-ray detector and expressed as an atomic percentage of the respective elements present. From the spectrum obtained, the ratios of calcium to phosphorus (Ca/P) and carbon to phosphorus (C/P) were calculated.

2.4. Attenuated total reflectance-Fourier transform infra-red (ATR-FTIR) spectroscopy

The FTIR analysis was carried out using approximately 10 mg of powdered enamel samples weighed and placed directly into the instrument (Shimadzu IR Prestige 21); ATR-FTIR spectra were recorded at mid-frequency range (4000-400 cm^{-1}) at 4 cm^{-1} resolution. The chemical composition of the enamel was determined by comparing the recorded spectrum with the standard spectral library. A baseline correction was made before the interpretation of data. The percentage absorbance of elemental functional groups in the enamel matrix powder was recorded before and after radiation exposure. The carbonate: phosphate (CO^{2-}_3:PO^{3-}_4) functional groups ratio was calculated from the integrated area values of peaks obtained for the respective elemental composition in the FTIR spectra.

2.5. X-ray diffraction (XRD) spectroscopy

The XRD measurements of the enamel samples (10 mg) were performed with a Rigaku Mini Flex 600 laboratory diffractometer using Cu-Kα radiation (λ = 1.5406 Å) working at 40 kV, 15 mA. The diffraction patterns were recorded in a scan speed of 1θ per second at a rate of 1 s per step and within the 2θ angle range of 10–60°. Phase identification was carried out from the diffraction pattern

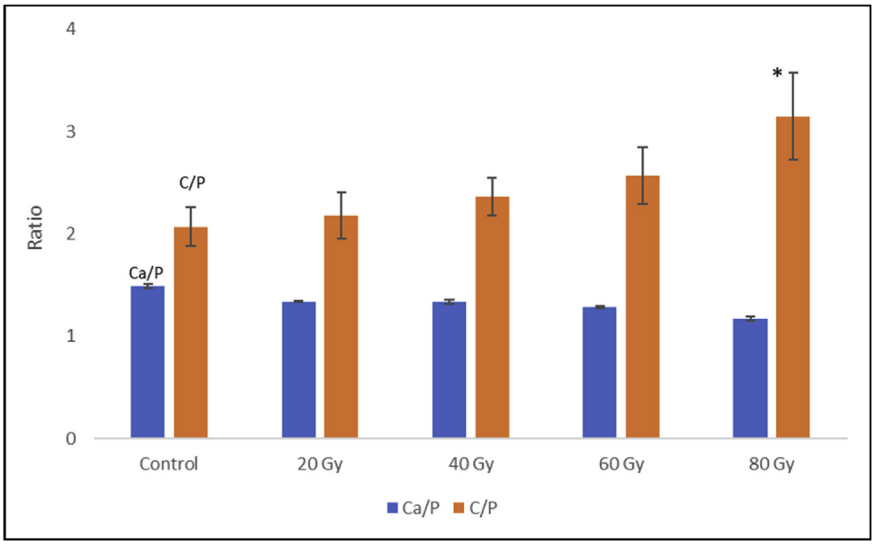

Fig. 3. Control (n = 22) compared with 20 Gy (n = 3), 40 Gy (n = 3), 60 Gy (n = 3), 80 Gy (n = 3). Compared to control, the samples exposed to 80 Gy showed significant increase ($P < 0.05$) in the C/P ratio and decrease in the Ca/P ratio.

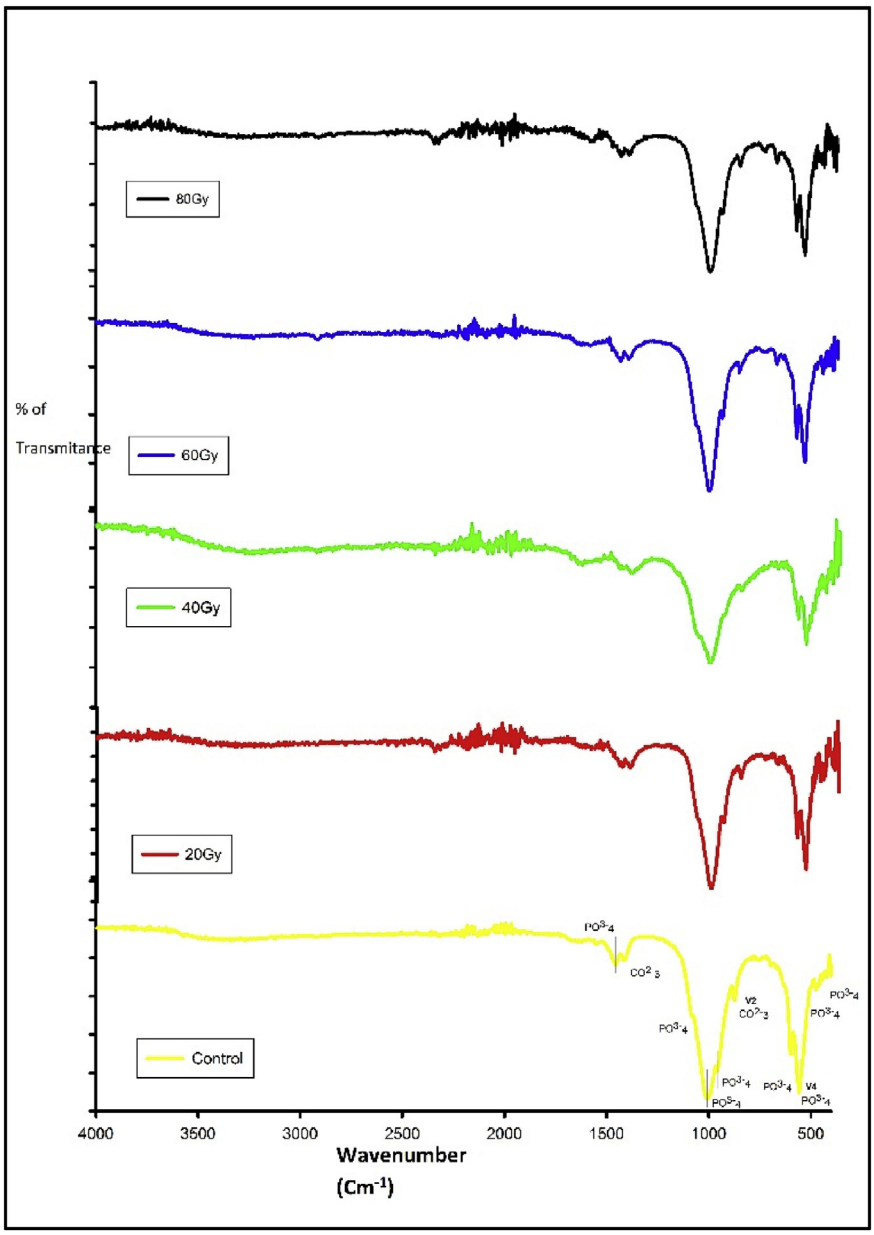

Fig. 4. FTIR spectra of teeth enamel powder; unexposed control (n = 1) and exposed (n = 4) samples using a 400–4000 nm wavelength scan.

with reference to a standard hydroxyapatite diffraction pattern (JCPDS 76–0694) and the crystallite size was calculated using Scherer's formula (1).

$$Particle\ size = 0.9\gamma / d\cos\theta \quad (1)$$

Where, $\gamma = 1.54060A^{\circ}$

$\theta = 2\theta$ angle range in the X axis (Fig. 5).

d = The full width at half maximum (FWHM) intensity of the peak (expressed in Rad).

3. Results

3.1. EDS spectroscopy; carbon: phosphorous (C/P) and calcium: phosphorous (Ca/P) ratios

The ratios of C/P and Ca/P were calculated from the atomic percentage values of the respective elements in the EDS spectra. A significant increase in the C/P ratio was observed for irradiated samples ($P < 0.05$) in the 80 Gy group compared to the control (Fig. 3). The average Ca/P ratio in control samples was 1.49 ± 0.11, typical of sound teeth enamel. The ratio decreased with radiation but the difference was not significant compared to the control ($P > 0.05$) (Fig. 2). Overall, this suggested variation in the organic and inorganic content of the enamel surface, with a dose-dependent deterioration of the elemental phosphorous composition, and a significant loss at a dose of 80 Gy.

3.2. FTIR spectroscopy

The FTIR spectra of teeth enamel powder from exposed teeth and control teeth are represented in Fig. 4. The integrated area ratio of the CO_3^{2-} v2 contour at 873 cm^{-1} by the PO_4^{3-} v4 contour at 557 cm^{-1} was calculated to obtain the carbonate to phosphate ratio in the samples (Table 1). A dose-dependent increase in the carbonate: phosphate ratio was seen at 557 cm^{-1}, indicating a greater

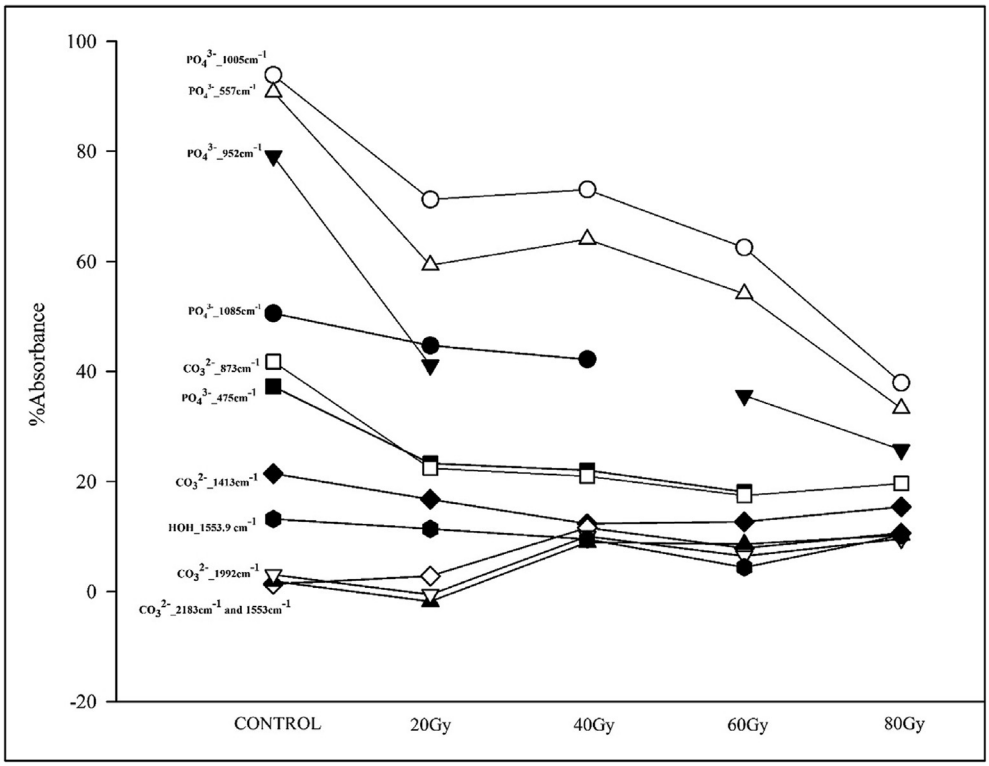

Fig. 5. Percentage absorbance of elemental functional groups in the enamel matrix powder before and after radiation exposure.

Table 1
$CO^{2-}{}_3$:$PO^{3-}{}_4$ ratio was calculated as the integrated area ratio of the CO_3^{2-} v2 contour at 873 cm^{-1} to the PO_4^{3-} v4 contour at 557 cm^{-1}

Sample	CO_2^{-3}: PO_3^{-4}
Control	0.303
20 Gy	0.288
40 Gy	0.780
60 Gy	4.458
80 Gy	2.093

extent of deterioration of phosphate compared to the carbonate functional group. In addition, the percent absorbance of the PO_4^{3-} v4 (at 557 cm^{-1}) contour decreased more than that of the CO_3^{2-} v2 (at 873 cm^{-1}) contour with increasing radiation doses. Also, phosphate v3 and v1 (at 1085 cm^{-1} and 957 cm^{-1}, respectively) bands disappeared after exposure to 20 Gy (Fig. 5).

3.3. X-ray diffraction (XRD)

The XRD graphs of unexposed control and exposed teeth enamel powder are represented in Fig. 6. In the control specimen, the spectral patterns were identical to the hydroxyapatite having consistent peaks with the standard hydroxyapatite pattern (JCPDS 76–0694). Loss of seven peaks at $2\theta = 20.14°$, 25.36°, 22.81°, 25.31°, 28.6°, 32.9°, and 39.2° coordinates was observed in radiation-exposed samples (Table 2). In addition, flattened peaks were observed, indicating loss of crystalline properties (Fig. 6). The crystallite size of enamel samples (before and after radiation) was calculated from standard peaks and is represented in Table 3. As compared to control, the hydroxyapatite crystallite size of the radiation-exposed enamel surface increased with radiation dose.

4. Discussion

In EDS, derivation of atomic percentages of specific target surface areas on enamel helps in the quantification of their elemental composition. Atomic percentages of C, O, P and Ca were analyzed and the atomic ratios of Ca/P and C/P were calculated. Stoichiometrically, the Ca/P ratio is 1.67 for a pure form of hydroxyapatite and other bioapatites [19]. In the natural hydroxyapatite structure [$Ca_{10}(PO_4)_6(OH)_2$] in teeth enamel, the lattice structure allows incorporations of certain inorganic elements, which are absent in the pure compound [20]. However, the organic phase has a protein structure comprising collagen, laminin, matrix metalloproteinases (MMPs), etc [21]. Therefore, in control samples, the average Ca/P ratio was 1.49 ± 0.11, which is in agreement with previous studies [19]. The proportional decrease of the Ca/P ratio with increasing radiation indicated variation in the inorganic content of the enamel. An increase in the C/P ratio with radiation, and a significant difference with exposure to 80 Gy as compared to control samples, suggests that variation in the phosphorus composition was higher than the variation in the carbon content. However, in previous EDS studies on radiation-treated enamel, there was no modification observed in the concentrations of mineral content based on the values of Ca and P, emphasizing more of a decarboxylation and water lysis-mediated enamel degradation [22–24].

In previous studies, FTIR, Raman vibrational microspectroscopies were used for spectral bio-diagnosis of human tissues [25]. In the analysis of the hydroxyapatite crystal lattice in biological tissues, it is given that biological PO_4 v1 and v2 vibration peaks are capable of describing alterations in enamel structure. The quantitative description of the effects of hydrogen peroxide on enamel was made by calculating the area of the biological PO_4 v1. The biological PO_4 became wider when the treatment time or concentrations were increased. The biological PO_4 v2 was distorted at 20% H_3PO_4 concentrations [26]. We observed similar changes

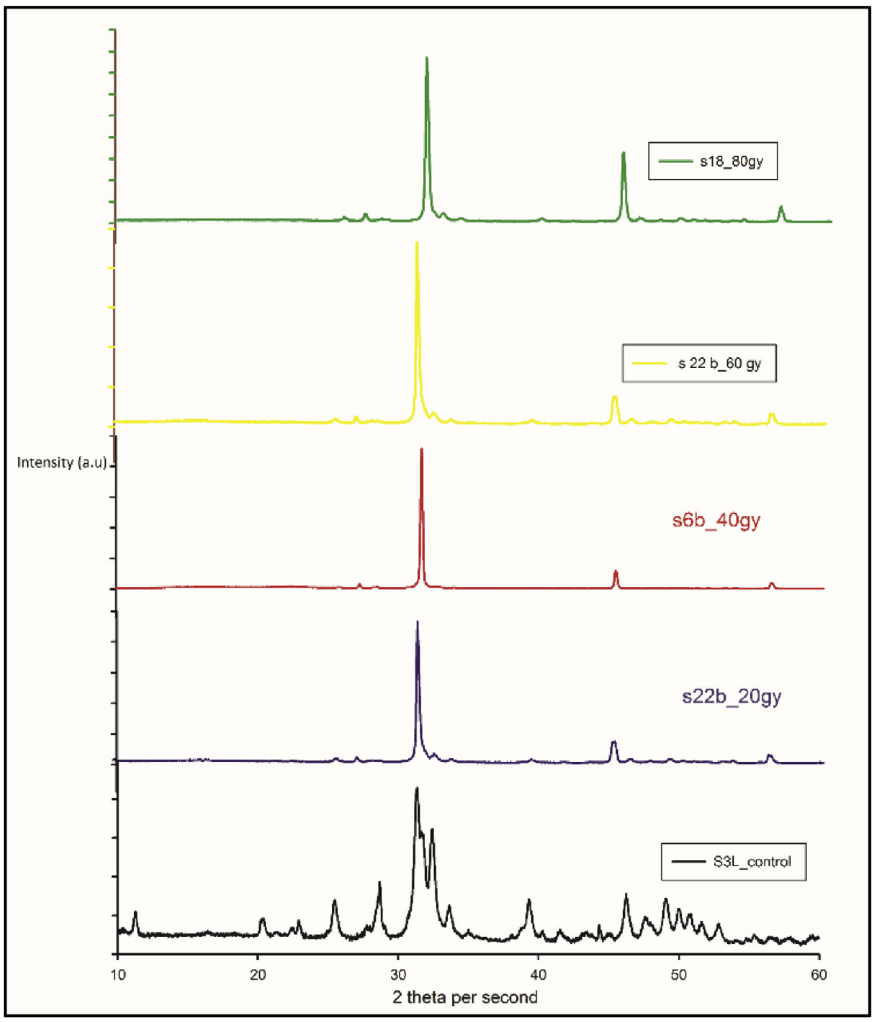

Fig. 6. XRD graphs of teeth enamel powder; unexposed control (n = 1) and exposed samples (n = 4).

Table 2
Peak values from the standard JCPDS (76–0694) file. Seven underlined 2θ peak coordinates disappeared in treated specimens.

Peak coordinates	Intensity value
20.14	(200)
25.36	(002)
31.28	(−221)
22.81	(−141)
25.31	(201)
28.6	(220)
32.9	(300)
39.2	(−162)
45.3	(043)
56.2	(034)

Table 3
Crystallite size of enamel samples (before and after radiation), calculated from standard peaks, FWHM values, and Scherrer's equation.

Sample	FWHM (2θ)	Crystallite size (nm)
Control	0.356	24.31
20 Gy	0.367	24.56
40 Gy	0.327	27.97
60 Gy	0.250	36.65
80 Gy	0.230	39.47

with respect to phosphate (V_2) and carbonate (V_4) groups wherein, a considerable shift with increasing doses of radiation was seen (Fig. 4). Two of the phosphate vibrations at 952 cm^{-1} (V_1) and 1085 cm^{-1} (V_3) disappeared after irradiation with 20 Gy (Fig. 5). However, our results did not indicate much variation in the carbonate composition as represented by the atomic ratio and the FTIR peaks. A similar observation was made in another study which observed comparatively more of a decrease in the PO_4^{3-} v1, v3 areas than the CO_3^{2-} v2 area, along with an increase in the carbonate: mineral ratio with radiation dose [16].

The crystalline phase, crystal physical properties and mean size of the crystalline particles of the teeth enamel surface defined by the XRD spectra are very useful for assessing teeth enamel ultrastructure. The average crystallite size in the specimens varies from ~12 nm (older age teeth) up to ~38 nm (younger age teeth) [27]. The absence of diffraction peaks in the radiation-exposed group signifies the deterioration of the crystal matrix structure. In addition, the control sample displayed sharpened peaks, indicating a stable crystalline structure while the radiation-exposed enamel spectra showed comparatively flattened peaks because of the transition to the amorphous state from the stable crystalline structure. Enlargement of the crystallite size from 24.31 nm in the control to 39.47 nm in the 80 Gy-exposed specimens (Table 3) indicates destabilization of the hydroxyapatite matrix due to softer and decreased mechanical properties, as compared to the smaller

crystals in sound crystalline enamel surfaces seen in control samples [28].

Interaction of the organic matrix with apatite crystals results from the electrostatic binding of collagen carboxylate sidechains and mineral surface phosphate groups through decarboxylation of the carboxylate sidechains, promoted by radiation, which precedes the formation of new calcium ion bridge phosphate groups. The mineral organic interaction is reduced and the development of CO_2 may induce microcracks in the hydroxyapatite, resulting in a roughened surface. Denaturation of the organic matrix caused by radiolysis would reduce the physical anchorage between enamel and dentin and the inner stability of dentin since it contains collagen fibers as well [29–32].

However, the predominant presence of inorganic phosphate groups and calcium appetites in the superficial enamel surface is under major risk compared to the organic content, which constitutes only 4% of the total elemental composition of the teeth enamel surface. Many previous studies have underplayed the impact of radiation exposure on the inorganic content, focusing mostly on mechanisms of RT-induced decreases in the organic content, further leading to tooth loss. Mature human tooth enamel is an acellular tissue, the majority of which is a natural carbonated hydroxyapatite (c-HAP) containing 96 wt.% of c-HAP as inorganic content, 4 wt.% of organic material and from 1 to 6 wt.% of water [33,34]. Our study observed predominant changes occurring in the enamel inorganic functional groups when subjected to radiation. As for the organic content analysis, the changes observed in carbonates in the FTIR and EDS analysis were relatively inconsistent. One reason for this could be the meager distribution of the organic content on the enamel surface, which might have hindered the inference from our experiments.

5. Conclusions

Three analytical techniques, EDS, FTIR and XRD, were used to analyze the effects of different doses of radiotherapy on the composition of permanent teeth enamel. These *in vitro* experimental observations provide important evidence towards teeth enamel delamination that may cause radiation caries as a direct effect of radiotherapy. Overall, there was significant variation in the functional groups of the hydroxyapatite crystal lattice constituting the teeth enamel, and loss of mineralization and crystalline properties, indicating alteration of the superficial enamel surface by radiation exposure. We recommend that further studies and treatment strategies are incorporated into *in vitro* study models to better understand the impact of RT on non-target tissues, and in the long run, to aid in an effective RT protocol.

Ethical statement

This study was approved by the Yenepoya University Ethics Committee (YUEC218/2017) bearing Protocol No: 2017/073 dated May 31, 2017.

Conflicts of interest

The authors state that there are no conflicts of interest in connection with this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.job.2019.10.002.

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