In vitro study of the composition and microhardness of the hard tissues from oral cavity submitted to gamma irradiation

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Introduction: Clinical Radiotherapy is one of the most important techniques for the treatment of malignant lesions of the head and neck, however, exposure to ionizing radiation can lead to systemic complications or sites during and after radiation treatment. Among these immediate local complications in oral cavity, it stands out the xerostomia and consequent oral mucositis. Regarding late complications produced by radiation, decay of radiation and osteoradionecrosis, both dose-dependent lesions, which mostrate a high level of incidence in recent decades (1-30%) and unwieldy, although these are presented after completion of treatment and under the influence of local factors. **Methodology:** The methodology proposed in this study aims to examine the direct effect of ionizing radiation after gamma irradiation of enamel, root dentin and jawbone samples, through the dose rate used in patients suffering with head and neck cancer. The samples were analyzed by microhardness surface analysis, scanning electron microscopy (SEM) and Fourier transform infrared spectrometry (FTIR) before and after irradiation**. Results and discussion:** The data were analyzed statistically with a significance level of 95% ($p < 0.05\%$), through the parametric Student's t-test for related averages and ANOVA statistical test, finding a statistically significant result ($p = 0.00$) for the four groups of samples. **Conclusion:** From the statistical results, it is concluded that the effect of gamma radiation on hard tissues of the oral cavity was highly significant regarded to physical, compositional and morphological properties.

Introduction

Cancer has constituted a public health problem, so its control and prevention must be prioritized in the world. Cancers develops with multiple stages over the years, so some types can be avoided by eliminating exposure to the determinants and contributing factors. If the malignancy potential is detected before the cells become malignant at an early stage of the disease, the treatment can be very effective and with high chances of cure[2]. Among the conventional surgical treatments commonly used in cancer treatment, other therapies were used like radiotherapy, widely used as definitive or adjuvant therapy for the surgical procedure, mainly in cancers of the head and neck[3]. However, high doses of radiotherapy in large areas, such as the oral cavity, upper jaw, mandible, and salivary glands may result in undesirable effects, of immediate or late onset, where osteoradionecrosis (ORN) has been considered to be the worst [4]. ORN is one of the most severe and serious oral complications of radiation therapy for head and neck cancer. Some authors state that, despite the improvement in oral care performed before radiotherapy, the incidence of ORN has increased significantly in recent years almost 1% to 30% per year [3]. It has been defined as the irradiated necrotic bone exposed, which does not heal over a period of three or six months without the presence of tumor remained or tumor recurrence. This pathological entity can be found both in the upper jaw region and in the mandibular region, with a high incidence rate in the region of the mandibular body and retro molar region documented in recent years[4-7]. The classic symptomatology of ORN consists of intolerable pain, pathological fracture of compromised bone, devitalized bone sequestration, fistulous processes, which produce inability for patients to eat normally. The onset of ORN in less than two years after radiotherapy is due to the high doses of radiation of more than 70 Gy that received the patients affected by head and neck cancer, and concomitantly to perform some surgical procedure after treatment with ionizing radiation. Radiotherapy reduces the proliferation of bone marrow, periosteal tissue and endothelial cells in the production of extracellular matrix components such as collagen type I, one of the most important constitutive proteins of bone tissue and root

dentin[8]. Among the risk factors for the development of ORN, one of them considered the most important by the authors is ionizing radiation, whose different modalities to develop this complications include: the total dose, amount of energy per photon, brachytherapy, irradiation field and dose fractionation. Although new modalities of radiotherapy such as Modified Intensity Radiotherapy (IMRT) have been developed, in which small volumes of the upper jaw and mandible receive high doses of radiation, there are still cases of ORN in the patients submitted to these In which the need for further studies to clarify the pathogenesis of ORN, specifically the direct and isolated action of ionizing radiation on the hard tissues of the oral cavity, and subsequently establish future modalities in the treatment and prevention of this deleterious effect of radiotherapy[4]. According to studies [3, 4], the ORN is unlikely to appear when the radiation dose is less than 70 Gy; but, other studies refer to an increase in the incidence of ORN in patients who received doses between 65 and 70 Gy, with an average dose of 66 Gy[3]. In view of the high incidence (1-30%) of the osteoradionecrosis[3, 4] that affects patients undergoing head and neck radiotherapy for the treatment of cancer, the present study aims to add a relevant data still unpublished in the literature. Osteorradionecrosis is a complication that decreases the survival of the irradiated patient, because of the risk that it can cause like a local infection and systemic processes, which considerably reduces the quality of life of the irradiated patient. Besides, osteoradionecrosis may require mutilating surgical treatment which decreases mainly oral and physiological functions. After the aforementioned, this study was proposed to better understand the mechanism of development of this complication by the effect of ionizing radiation, involving all the hard tissues of the oral cavity, with the purpose of determining in vitro, the direct actions of the gamma radiation as an isolated risk factor for the development of undesirable effects of radiation therapy, which may benefit, after further studies, the management of these complications of radiotherapy treatment in patients with head and neck cancer. Although a recent study[5] from the Biophotonics Laboratory of the Nuclear and Energy Research Institute - IPEN (São Paulo - Brazil) shows that, alone, the enamel submitted to ionizing radiation may not be more susceptible to demineralization than non-irradiated enamel observed in vitro. In the case of root dentin, which has higher organic content than enamel it was the most affected by gamma radiation compared to coronary dentin, which has a higher mineral composition, as demonstrated in the literature [6-8]. In the irradiated patient it is extremely important that the oral environment is adequate before the radiotherapy treatment and that proper nutrition and hygiene conditions are maintained during and after the protocol of ionizing radiation therapy. However, the difficulty of sanitizing the oral cavity occurs in individuals submitted to radiotherapy mainly due to the incidence of mucositis, muscle stiffness or trismus, and in the case of alveolar tissue, the presence of osteorradionecrosis as a dose-dependent deleterious effect, which may occur both during and After radiotherapy[3, 4].

Although some studies[9-21] report unfavorable results in the tissues of the oral cavity with greater organic content after radiotherapy treatment; However it is necessary to analyze the amount of mineral loss that occurs in the hard tissues submitted to gamma irradiation. Considering that it is indispensable to investigate preventive strategies that act in a more prolonged way in the irradiated tissues and that these studies are impracticable to perform in a patient submitted to the radiotherapy from the ethical point of view, there is a need to develop an in vitro protocol[14, 23 -27].

The determination of the effects caused by ionizing radiation, using the dose used in patients with head and neck cancer, in terms of variations in biochemical composition and surface microhardness of hard tissues [22-31], will in the future cause the possible inherent complications of radiation to be reduced or elimination by dose reduced fractionation protocols or preventive procedures, with data obtained from in vitro studies [31-40].

Therefore, this study aims to determine, through physical, morphological and compositional properties evaluation, the direct effect of gamma radiation on the hard tissues of the oral cavity by surface microhardness analysis, Scanning electronic microscope (SEM) and Fourier transform infrared spectroscopy with attenuated total reflection technique (FTIR-ATR).

Material and Methods

For the present study were selected fifty healthy third human molars and two swine mandibles in order to obtain four groups submitted to analysis: enamel slabs (n= 50 samples), root dentin (n=49 samples), mandibular body (n= 51 samples), retromolar region (n=49 samples). This study was approved for the ethical committee in human and animal for scientific studies of Energy and Nuclear Research Center, Sao Paulo, Brazil.

Cutting and obtaining blocks of dental enamel and root dentin

In the present study, 50 healthy human third molars were selected from the teeth bank of the University of São Paulo School of Dentistry (USP), which were kept in Thymol solution (concentration 1 g / L) for 48 hours and refrigerated at 4 °C. After the initial decontamination of the teeth, they were placed in a container with distilled and deionized water to begin the separation of the crowns and roots, in order to obtain the blocks of dental enamel and root dentin. A high-speed diamond-tipped instrument was used, making cuts at the level of the cemental enamel junction (CEJ). Afterwards, the blocks were demolished, that is, all the remaining organic tissue was removed, using curettes for scaling and root planing (Millenium 13 MC and 34 straight, curve 28, Millenium 28 with fine point, São Paulo, Brazil) . These were then subjected to ultrasonic cleaning (Unique Ultrasonic Washer - Thomton, Unique Ind. And. Com. Ltda., São Paulo, Brazil) for a period of 30 minutes divided into two cycles of 15 minutes each one to eliminate organic debris not completely removed. The blocks were placed in 200 ml volume beakers with distilled and deionized water and placed in the center of the washer tub, previously filled with water, and after the first ultrasonic washing cycle, the water was discarded and it was exchanged of the containers to start the second washing cycle. After the second wash cycle, the water in the containers was again discarded, finally 20 ml of distilled water was poured and the beakers were covered with aluminum foil and stored under refrigeration at 4 ° C for further macroscopic analysis. For the macroscopic analysis of the blocks obtained, inclusion and exclusion criteria were established, which included the crowns and roots free of visible defects and white spots of both hard tissues, excluding the blocks that did not comply with the aforementioned characteristics. Moreover, he crowns and selected roots were fixed on an acrylic sheet approximately 4 cm wide by 5 cm in diameter, using wax yellow sticky stick placed in a lamp and heated until it melted and was transported to the acrylic sheet by a metal dripper. A drop of wax was placed for the parallel positioning of one of the edges of the crown to the lateral edge of acrylic sheet, and finally the sides of the crown were fixed. Once the crown was fixed to the blade, it was cut in a transverse and horizontal direction in the manual cutter (IsoMet. Buehler, *São Paulo*, Brazil), with a fine-grained aluminum diamond disk (Struers, São Paulo, Brazil) . The crown to be cut was positioned almost parallel to the larger arm of the equipment and the disk was directed for the sectioning of the crown in a transverse direction, with a distance of approximately 3 mm x 3 mm between cut and cut until reaching the basal part of the crown and were obtained the blocks of dental enamel.

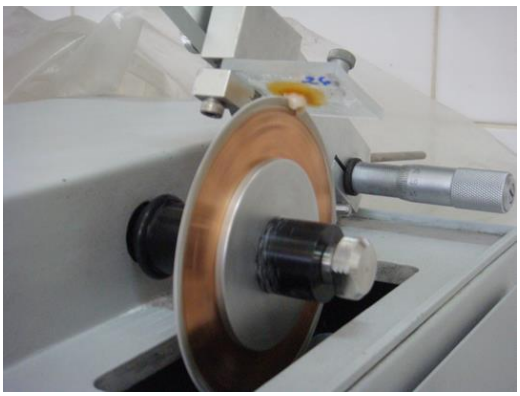


Figure 1. Cutting with fine-grained aluminum disc to obtain blocks of dental enamel

For the cutting of the roots, the procedure similar as enamel samples obtained, previously described , was carried out with previous fixation of the root and subdividing in three imaginary lines the frontal face of it in transverse and horizontal direction. The root dentin blocks of approximately 3 x 3 x 1,2 mm were obtained.

Polishment protocol for enamel and root dentin samples

Polishing of dental enamel blocks

Once the enamel and root dentin blocks were obtained, they were submitted to manual polishing to analyze the initial

surface microhardness. The blocks were mounted on the acrylic sheet, fixed with sticky wax as previously described. In the case of dental enamel blocks, the enamel surface was first fixed to the acrylic sheet in order to polish the remaining dentin and obtain a plane surface completely parallel to the edges of the blade in use. Then the blocks were polished in the Politriz, in which grit discs of different grains were used: started with a 400-800 disc of coarse grain under refrigeration for 15 seconds with a turning speed of 100 rpm, then a 600-1200 disc under refrigeration for 10 seconds with a spin speed of 100 rpm. Thereafter, the 2500 discs were used for 10 seconds and at a speed of 100 rpm, and to finish a 4000 fine grain disc was used during the time and speed previously described. The polishing was completed with the ultra polishment of the blocks with 1 µm diamond solution (Buehler, *São Paulo*, Brazil) without refrigeration, poured into the felt disc and executed at a speed of 300 rpm for 1 minute.

The procedure described above was performed for the dental enamel surface, which was turned upwards and consequently the previously polished dentin was turned to the acrylic sheet, fixing it with sticky wax on the lateral edges with the precaution of avoiding contact between the wax and the block.

At the end of the polishing of the blocks, samples obtained from dental enamel were subjected to ultrasonic lavage for 40 minutes subdivided into 5 cycles of 8 minutes each, and the distilled water was discarded in each cycle. The sample beakers were filled with distilled and deionized water and stored for 24 hours for subsequent fixation of the samples on the acrylic sheet.

The root dentin blocks were polished as well as enamel blocks. Both surfaces of the root dentin were polished until a flat surface of approximately 3 x 3 x 1, 2 mm was obtained, in politriz and with the sandpaper discs of 400-800, 600-1200, 2500 and 4000 according to the previous protocol. Finally, they were subjected to ultra-polishing with 1 µm diamond solution, without refrigeration, at a speed of 300 rpm for 1 minute.

At the end of the polishment of the blocks, the samples obtained from root dentin were submitted to ultrasonic lavage, as described for enamel samples.

Preparation of swine mandibular bone samples

Swine jaws were selected with 15 cm as length and 10 cm as width, provided by *Raia* Frigorific (*Carapicuíba, São Paulo*, Brazil). The jaws were washed with plenty of distilled and deionized water and then stored in sterile containers in freezer for maintenance of biological tissues not removed at a temperature of -88 ° C.

The remaining soft tissue removal process was carried out in the Laboratory of preparation of biological samples of the Lasers and Applications Center, such as muscular fasciae, muscles and ligaments belonging to the tissue structure of the mandibular bone. It was used a scalpel sheet n° 15, a scalpel handle n° 3, and a toothless forceps for pressing the tissue and final excision. A thorough washing with distilled and deionized water was performed after complete removal of the remaining tissues, obtaining a clean and homogeneous surgical piece superficially. After each surgical procedure, the jaws were wrapped in a sealed plastic envelope and stored under a temperature of -88 ° C for preservation of the surgical pieces.

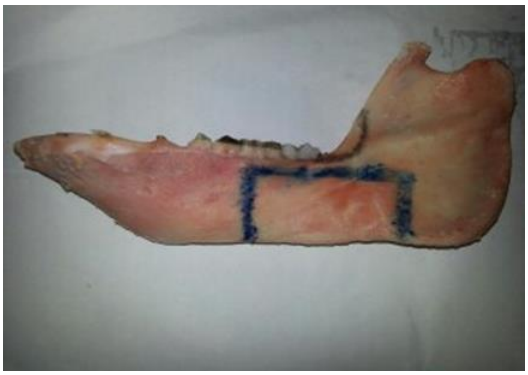


Figure 2. Delimitation of the medial region of the mandibular body on the left side to obtain blocks of 3 x 3 x 1, 2 mm

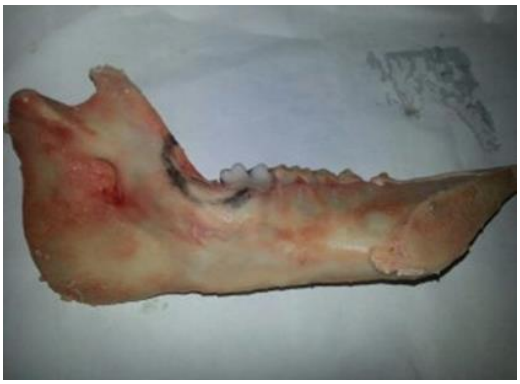


Figure 3. Delimitation of the internal region of the left retromolar trigone to obtain blocks of 3x3x1.2 mm

The surgical specimens were subdivided into two halves for better management of the hemimandibles on both the right and left sides. The blocks were obtained from the region of the mandibular body, through manual cutting with a low rotation motor, with an aluminum diamond disc and a metallic carburundum disc, through which abundant irrigation with distilled water until the final block was obtained in order to decrease the thermal effect after cutting. In the case of the blocks of the retro molar region, the previously described procedure was performed.

After the blocks were obtained from both groups, they were submitted to ultrasonic lavage to remove the remaining organic tissues. The blocks were placed in sterile beakers with distilled and deionized water, followed by washing for 5 cycles of 8 minutes each, and finally stored under a temperature of -88 ° C until further analysis.

Mandibular body blocks were submitted to vertical and transversal cutting in the Manual Cutter (IsoMet, Buehler, *São Paulo*, Brazil) to obtain samples smaller than 3 mm x 3 mm x 1.2 mm. They were attached to the acrylic blade with sticky wax, with the edges of the bone parallel to the edges of the blade. Cutting started at the outermost edge of the block and continued to the inner edge, taking as a distance between cut and cut 3 mm x 3 mm x 1.2 mm. Thereafter, samples were undergone to ultrasonic lavage for two 15 minute cycles.

Afterwards, the blocks were fixed again to the acrylic sheet with sticky wax and the manual polishing of the same was carried out in politriz. Polishment protocol was done as well as described previously for enamel and dentin samples. Therefore, ultrasonic lavage was performed to remove the remains of the diamond solution. The blocks were stored in a beaker with distilled and deionized water, capped hermetically and cooled to 4 ° C for 24 hours.

For retromolar region preparation and Polishment samples were carried out at the same way as enamel, dentin, and body mandibular samples. Samples were fixed and submitted a surface microhardness initial analysis (baseline).

Analysis of Initial Surface Microhardness (Baseline)

Samples obtained from the four study groups and fixed on the acrylic sheet were submitted to the Initial Surface Microhardness (ISMH) analysis in order to obtain a quantitative evaluation of the homogeneity on the surfaces of the samples after polishing. Thus, for ISMH analysis (Baseline), a microdurometer (Shimadzu HMV-200, Japan) with Knoop indenter was used which was programmed to apply a load of 245.2 mN (HK0,025)) for 10 seconds in case of dental enamel samples, and a load of 98.4 mN (HK0.01) for 15 seconds (s) for root dentin, retro molar trigone region and mandibular body samples.

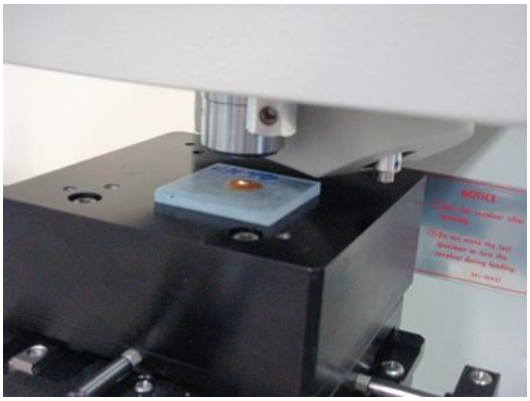


Figure 4. Analysis of Initial Surface Microhardness through Knoop indenter. The sample was centered perpendicular to the indenter

Each sample from each group was placed in the microdurometer and kept unmoved throughout the analysis. For each sample, 15 indentations were subdivided into three rows with 5 indentations each one, which were separated at distances of 100 µm from a regular border, which was previously selected and marked for future reference. The distance between row and row was 200 µm. After these baseline indentations, the mean and standard deviation of the 15 indentations of each sample were calculated to verify if it was homogeneous for the purpose of inclusion criteria. Scatter plots were drawn in Microsoft Excel (Microsoft Office, 2010) for each group, from this it was possible to make an accurate selection of the samples to be irradiated.

Sample irradiation

The samples were submitted to gamma irradiation in the Cobalt-60 Irradiator (GammaCell, Co^{60}) at the Radiation Technology Center (CTR) of the Nuclear and Energy Research Institute (IPEN - CNEN / *São Paulo*, Brazil) with the protocol

conventional irradiation used in patients with head and neck cancer. Samples were placed in Petri dishes of resinous material for cultivation, and then they were submerged in cotton fields humidified with a solution of Thymol diluted in distilled water (0.641 g / 1 liter of distilled water) in order to maintain the humid environment and prevent the proliferation of fungi.

Irradiation of dental enamel samples

Dental enamel samples were placed in Petri's plaques submerged in cotton humidified with the Thymol solution and distilled water. These were distributed in 5 samples per Petri's plaques.

Then, the irradiation protocol was initiated in the Cobalt-60 Irradiator (GammaCell Source, Co60) with a dose rate of the equipment from March 2016 of 903 Gy / h, a traffic dose of 0, 76 Gy, and finally a dose rate of 2 Gy / 4.94 seconds. Irradiation was performed twice daily (4 Gy per day) at a 8 hour interval for each irradiation for 5 days until a weekly dose of 20 Gy was completed. After the second irradiation of the day, the samples were submitted to irrigation with thymol solution and distilled water and then stored under refrigeration at 4 ° C.

Irradiation of root dentine samples

Root dentin samples were submerged in cotton, humidified with the thymol solution and distilled water and placed in Petri's plaques. These were subjected to gamma irradiation in the Cobalt-60 Irradiator (Gammacell Source, Co^{60}) with the characteristics previously described. Samples received a fractionated dose rate of 2 Gy / 4.94 s twice daily, completing a dose of 20 Gy weekly. The total irradiation dose was 72 Gy. Samples were rehydrated after the second irradiation time on the day.

Irradiation of mandibular body samples

Mandibular body samples were placed in Petri' plaques distributed in subgroups of 5 samples per plate, which were submerged in humidified cotton to maintain the humid environment and inhibited fungal growth. These were subjected to gamma irradiation at the Cobalto-60 Source (Gammacell, Co60) at a fractionated dose rate of 2 Gy / 4.94 s twice daily until a weekly dose of 20 Gy was completed, and a total dose of 72 Gy. After the second irradiation time, the Petri's plaques were rehydrated and stored under 4 ° C refrigeration.

Irradiation of retromolar trigone region

In the case of retromolar trigone samples, the previously described procedure for root dentin, dental enamel and body mandibular samples was performed.

Figure 5. Cobalto-60 Gammacell irradiator source. Dose rate: 2 Gy / 4.4 seconds, received twice daily (TOTAL DOSE: 72 GY)

Figure 6. Placement of Petri´s plaques with mandibular body samples before were submitted to gamma irradiation

Scanning electronic microscope analysis (SEM)

Four samples per group were selected to be evaluated morphologically by Scanning Electron Microscopy (SEM). At first the samples were treated and prepared (initial conditioning) physically for later analysis in the SEM. Different concentrations of ethanol were chosen for the purpose of dehydrating each sample included on an increasing scale: 10%, 30%, 50%, 70%, 90%, 96% and 100% for 15 minutes in each solution. Samples were examined in the Scanning Electron Microscope TM 3000 Tabletop Microscope (Hitachi, Japan) of the Materials Technology Center at IPEN / CNEN - *São Paulo*, Brazil, with a voltage of 15 Kv and Analy (EDS) for higher resolution

of the image with a broad specificity of the surface to be analyzed. A conductive, double carbon adhesive tape was used to facilitate the fixing of the sample to the SEM sample port.. For each sample analyzed forms obtained at least four images in different predetermined increases: the first analysis was developed with a magnification of 50 X for analysis of the general surface of the samples, later, the magnifications were increasing of 500 X, 1000 X , 2000 X, 4000 X. The structural and morphological characteristics of each hard tissue after undergone to gamma irradiation were observed, both the effects on the surface mainly and other regions; In addition to the analysis of the control group (without irradiation) in order to evaluate the homogeneity and initial characteristics of the normal pattern of biological tissues.

Attenuated total reflection – Fourier transform infrared spectroscopy analysis (ATR – FTIR)

The irradiated and non - irradiated samples were analyzed by Fourier Transform Infrared Spectroscopy on the FTIR (Perkin - Elmer, 100) infrared spectrometer of the IPEN / CNEN Radiation Technology Center (CTR), *São Paulo,* Brazil, whose spectra Were obtained by attenuated total reflection (ATR), in a frequency range of 4000 cm $^{-1}$ to 650 cm -1, and an acquisition accuracy of 4 cm $^{-1}$. A greater number of scans were used following the latest literature reports[32] to obtain hard tissue spectra of the oral cavity, completing a total of 80 scans with background subtraction (BG). The crystal that made up the equipment for the analysis was the zinc selenide (ZnSe), in order to obtain a biochemical, qualitative and semi-quantitative analysis of the organic and inorganic content present in them. Samples were detached from the acrylic slides and positioned above the ZnSe crystal to obtain the spectra generated by the Fourier transform. Immediately, after the samples were removed and centrally positioned on the crystal, the adjustment against the crystal was followed using a specific torque force of 130 N, which allowed an accurate and accurate analysis of the samples.

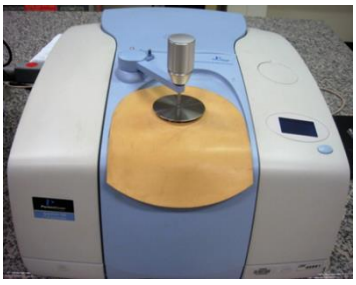


Figure 7. ATR-FTIR spectrophotometer for attenuated total reflection analysis of the samples.

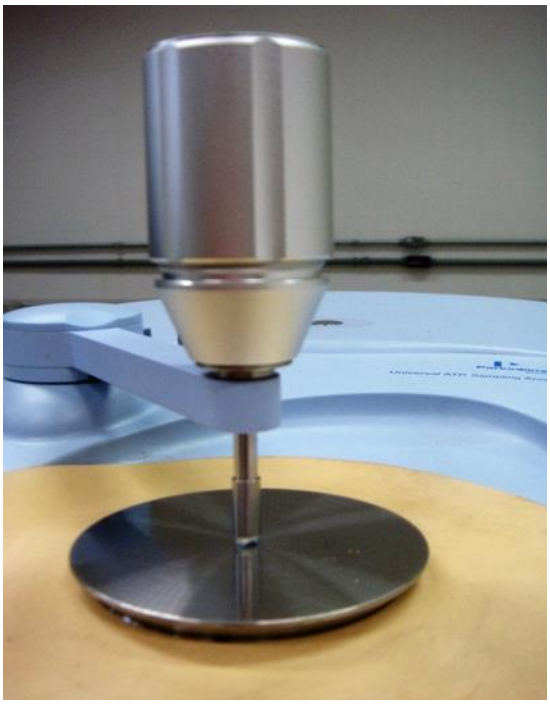


Figure 8. Analysis by attenuated total reflection of the samples under study, with the application of a force of pressure of 130 N.

Statistical analysis

For the statistical analysis was used the Statistical package for Social Sciences (SPSS) version 18. For widows. To analyze the mea values of surface microhardness before ad after irradiation, it was used t student test I order to compare if there was differences between initial and final mea value I each group. I order to analyze the mea value between group (inter- group), it was used anova ad turkey test, with a level confidence 95%($p < 0,05\%$)

Results ad discussion

i. Scanning electronic microscope analysis (SEM)

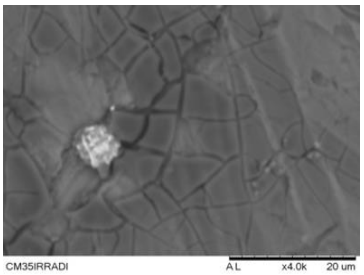


Figure 9. Image from body mandibular samples after gamma irradiation 4000x (total dose = 72 Gy).

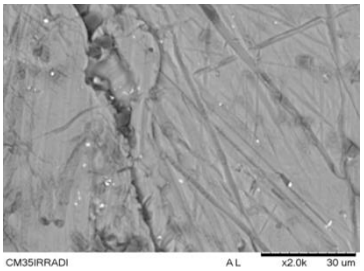


Figure 10. Image from body mandibular samples after gamma irradiation with 2000x magnification glass.

In pictures 9 and 10, mandibular body images show the deleterious effects caused by gamma irradiation on the surface of the hard tissue. The fractures, perforations and fracture lines of the samples submitted to irradiation are presented, distinguishing the different degrees of involvement in each increase, showing the displacements of the fragments, as well as the loss of continuity of the tissue in relation to a surface smooth, flat and without bumpy or perforated areas. In the last image, there is progressive fragmentation around a central nucleus, with separation of the forms, with the appearance of a comminuted fracture of the surface of the mandibular body sample. In addition, it is the peculiarity of this sample to show different types of cracks with reduced depth and a tenuous line of central fracture.

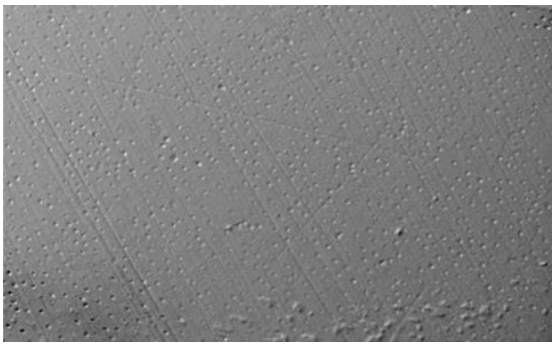


Figure 9. Image of the sample of the retromolar trigone region before gamma irradiation, after mechanical polishment, in which a smooth and uniform surface corresponding to apparently healthy tissue at the macro-regional level can be observed, with an increase of 500 X.

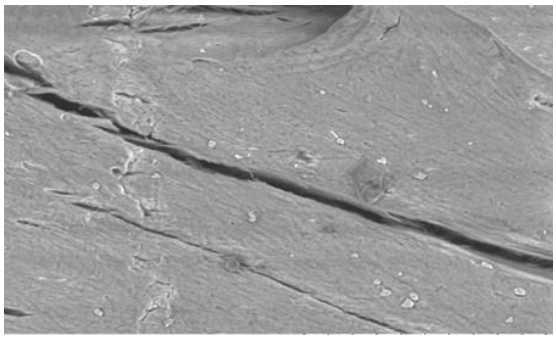
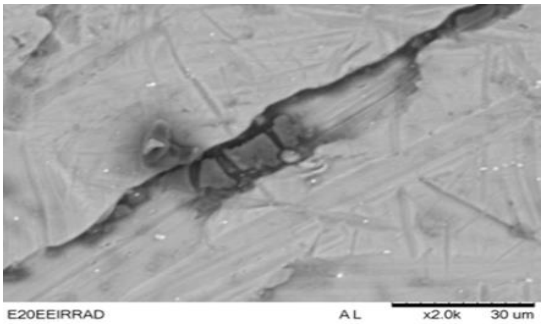


Figure 10. Image from the retromolar trigone region sample after total dose of gamma radiation (72 Gy). The image shows a marked disorganization of the analyzed surface, presenting a considerable length of crack, in addition to smaller cracks around the larger crack, whereby the radiation effect at the irradiated site is considered to have a greater deleterious effect in a LIcation compared to other surface locations. On the other hand, the instability generated by gamma radiation leads to further destruction of the tissue with loss of surface hardness.



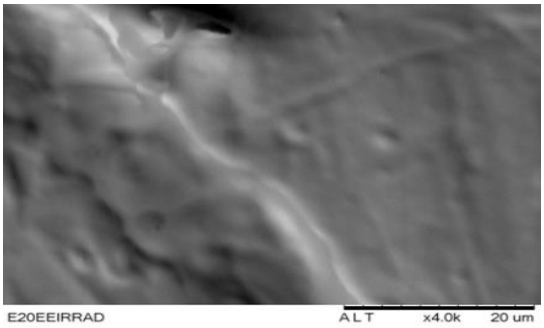
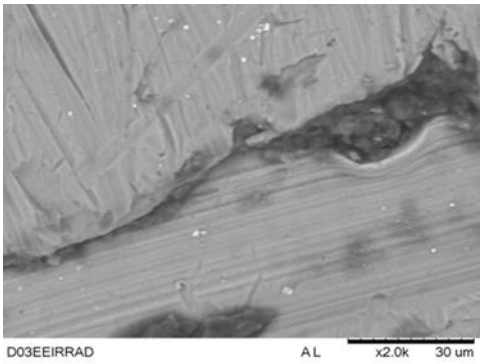


Figure 11, 12. Images from dental enamel samples after gamma irradiation shown at different magnifications in which a clear disintegration and deterioration of the enamel surface is observed, with the presence of cracks and fracture lines around the central surface.. In the first magnification 2000 x, it is possible to distinguish a crack of longer length at the surface level, in an irregular and bumpy shape, because the gamma radiation has altered enamel's structure integrity, also observing fragments of the hard tissue involved. Finally, the image 11 shows, a marked fracture line is observed on the surface, of considerable length, highlighted in a larger increase (4000 x).



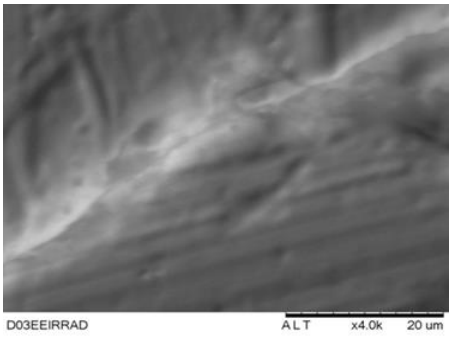
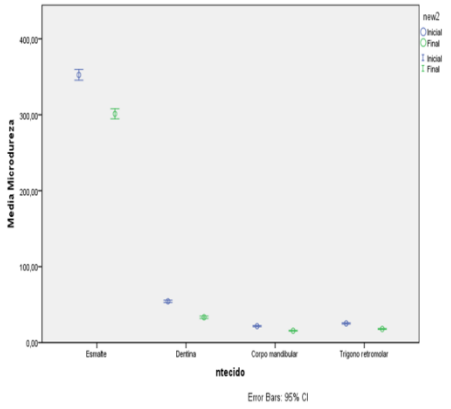


Figure 13, 14. Images from root dentin samples after gamma irradiation. At a magnification glass of 2000 x was observed a line of fracture stands out, with slight displacement of the edges and loss of continuity of the mineralized tissue, which again represents the involvement In another angle of the surface of the analyzed dentin block, obtaining greater details by use of an increase of the lens of the greater microscope. Finally, in the last figure, we observe along the main axis of the sample a crack that extends over most of the surface of the sample, being visualized in the topographic image mode and clearly detecting the deleterious effect caused on the irradiated block, with a 4000x magnifying glass.

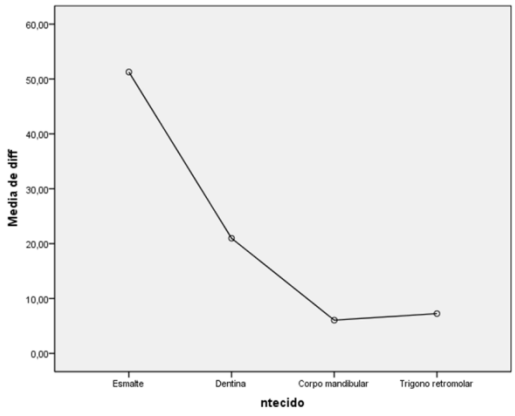
Surface microhardness analysis

After surface microhardness analysis of tissues before and after gamma irradiation by simulating an in vitro model of a patient affected by head and neck cancer, results show, a decrease in enamel ad root dentin hardness, corresponding to the results obtained by other studies[14,16,17,18].The results were grouped and normality, homogeneity and independence tests were performed to establish the statistical models to be used, in this case, the student's t-test of the related means was used for each study group, obtaining a value of p = 0.00, by which the hypothesis of nullity was discarded, disassembling that the ionizing radiation has a significant effect on the hard tissues of the buccal cavity, specifically, a deleterious effect with respect to the surface microhardness. In the other hand, root dentin group showed a slight superiority were detected in the last results when compared to the other tissues of greater organic content, such as the mandibular body and retromolar trigone region. Finally, the mandibular body group and the retromolar trigone region showed results very close when compared mean final results between them, whereafter it will be observed that there were no statistically significant differences, as showed in graph 1 considering these results as a similar effect exerted by gamma radiation on both tissues.



Graph1. T student test for analysis mean values of initial and final microhardness (level confidence: 95%)

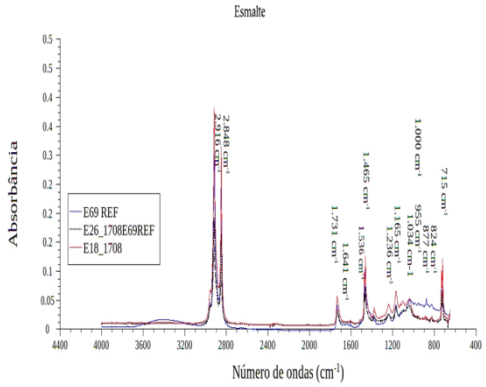
Enamel samples presented a higher mean value of difference between initial and final surface microhardness, probably it demonstrates statistically a greater involvement of enamel surface by gamma radiation, obtaining much lower values of final microhardness when compared with initial values as showed in graph 2 after ANOVA / Tukey statistical test was applied. Regarding dentin, a lower mean value was obtained when compared to enamel and superior when compared to mandibular bone regions. According to the recent literature[14, 15, 16], dentin was the hardest tissue of greatest involvement and degree of destruction by gamma radiation, considering its organic composition and a higher water content with respect to dental enamel, besides other local factors like proteinases and metálo-proteinases activated during the destructive and protein degradation process. In the present study, root dentin presented a lower rate of involvement when compared to enamel, although some studies[15, 16, 17, 18, 19] presented similar results with this study, but with a certain dose threshold (doses up to 30 Gy) presented a greater hardness enamel loss than in higher doses). The results presented in this study regarding the dental enamel would require a further revision and subsequent studies to be carried out on the direct effect of gamma radiation, be it the application modality, on human tooth enamel and the consequent loss of tissue hardness, with consequent physico-chemical disorganization and subsequently a rapid advance in the underlying tissues. Radiation cavities are characterized by the rapid advancement and destruction that it produces in the teeth of patients undergoing radiotherapy, and the fact that it requires a treatment of high complexity and difficult to manage and the physical and psychological sequelae in these patients, is possibly justified due to the fact that radiotherapy exerts a deleterious effect on tooth enamel, as demonstrated in this study.



Graph 2. Mean differences in the surface and initial microhardness values of the study groups

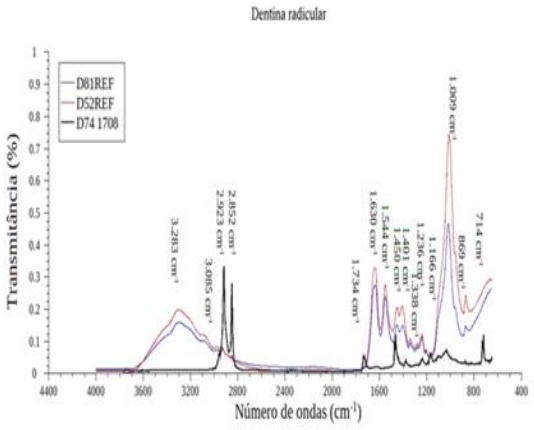
Fourier transform infrared spectroscopy (FTIR) spectroscopy with the attenuated total reflection technique (ATR)

Basically, dental enamel consists of polyhydroxyapatite (97%), protein (1%), being ameloblastine, enamelin, amelogenin, and water (2%). The absorptions in the infrared region of analyzed material converge to this constitution, once the presence of the phosphate and hydroxyl group, which are the functional groups constituting the polyhydroxyapatite, amide, carboxylic acid, oxygen and carbonic chain groups are the material-forming proteins, and the hydroxyl group at 3,425 cm $^{-1}$ is related to the hydroxyl that interacts through weak bonds of hydrogen bonds, such as water. Thus, the analysis of the composition converges to the information presented in the literature [41, 43, 44]. These bands show that there was a biochemical alteration of the material as a function of the irradiation process. The band related to amide II becomes more evident and the one related to polyhydroxyapatite becomes more intense, suggesting that the substance that was aggregating the different substances in the material may have degraded, evidencing polyhydroxyapatite. (graph 3)

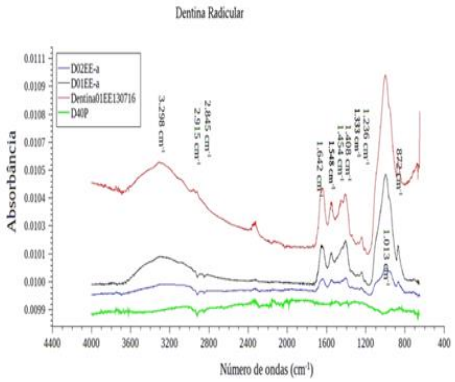


Graph 3. Spectrum of absorption from enamel samples before and after gamma irradiation in the medium spectral infrared region

On the other hand, root dentin consists of 18% organic material (collagen type I, lipids, citrates, glycoproteins and proteoglycans), 12% water and 70% inorganic material (polyhydroxyapatite and calcium phosphates). Spectral analysis showed organic composition of the material showing the amide functions (I, II and III) characteristic of the presence of collagen type I, the hydrocarbon base of the organic chain containing these groups. Spectrum absorption showed a comparative plot between the spectra of the reference samples (D81REF, D52REF) and the gamma irradiated sample with a total dose of 72 Gy (D74 1708). It is observed a change in the spectrum of the irradiated sample when compared to the reference spectra, which shows the disappearance of bands related to the presence of amide (I, II and III) and polyhydroxyapatite and bands are intensified at 2,923 cm $^{-1}$ and 2852 cm $^{-1}$. The band at 1734 cm $^{-1}$ is relative to the presence of peroxide. This behavior evidences the degradation of the material when exposed to ionizing radiation, where there is deterioration of collagen and polyhydroxyapatite; the presence of peroxides evidences the generation of highly reactive oxygenated products that are directly responsible for the degradation (graph 4). In the case of the second graph the spectra obtained from the samples submitted to gamma irradiation with total dose of 72 Gy are presented. Spectral similarity is observed in relation to the reference samples, but the bands at 1450 cm $^{-1}$ and 1336 cm $^{-1}$ disappear, suggesting that the irradiation process breaks down the carboxyl group of amides that constitute the organic part of this material. The same considerations of the previous result are pointed out in this case, evidencing the effect of the radiation in the material, although samples were submitted under same conditions and irradiation period, however, there is a great decrease in the intensity and absence of absorption bands in sample D40, evidencing a important change in the material. (graph 4)

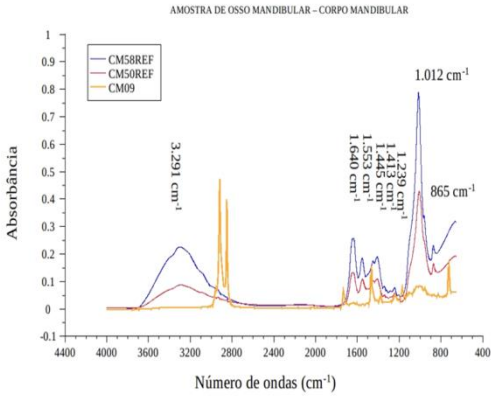


Graph 4. Spectrum of absorption from root dentin samples before ad after gamma irradiation in the medium spectral infrared region.



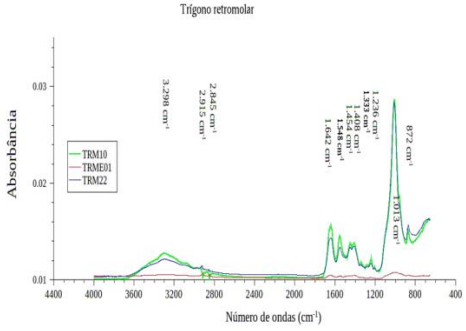
Graph 5. Spectrum absorption from root dentin samples after gamma irradiation I the medium spectral infrared region

The mandibular body consists of 65% of inorganic material (polyhydroxyapatite and calcium phosphates), 25% of organic material (type I collagen fibers) and 10% of water, with a compositional similarity to dentin [42, 44]. However, the presence of the bands at 1445 cm^{-1} relative to the -OH group of the carboxylic acid, 1336 cm^{-1} relative to the -CO group of the carboxylic acid and 1,239 cm^{-1} relative to the -NH (amide III) group are weak and less evident than in dentin. The plot (graph 5) compares the spectra of the non-irradiated samples (reference group) and the sample exposed to the total dose of 72 Gy. It is possible to observe the complete alteration of the spectrum of the irradiated sample when compared to the reference spectra, with the disappearance of the bands in 3,291 cm^{-1}, 1,643 cm^{-1}, 1,553 cm^{-1}, 1,413 cm^{-1}, 1,012 cm^{-1} And 865 cm^{-1}. The new bands at 2,923 cm^{-1}, 2,852 cm^{-1}, relative to the hydrocarbon chain of the organic constituents, appear at 1745 cm^{-1} relative to the OH-group of the carboxylic acid, at 1734 cm^{-1}. 1.336 cm^{-1} relative to the -CO group of the carboxylic acid and at 715 cm^{-1}, due to the out-of-plane angular deformation of the OH group bonded by hydrogen bonds of the phosphate material. All this change shows the degradation not only of the organic part that forms the material, but also the breakdown of the inorganic part, with strong oxidation action caused by the presence of highly reactive substances and oxidants such as peroxide.



Graph 6. Spectrum absorption from body mandibular samples before ad after gamma irradiation I the medium spectral infrared region

Finally, in the case of the retromolar trigone region, which has a composition similar to that of the mandibular body, but with a lower percentage of inorganic material (polyhydroxyapatite and calcium phosphates) and a little more organic material (type I collagen fibers) And with similar amount of water. In the graph 7 the spectra of the samples submitted to gamma irradiation (TRM10, TRME01, TRM22) are analyzed by attenuated total reflection (ATR-FTIR) analysis. It is observed a similar behavior between the bands obtained in each spectrum, and the intensity of the absorption peaks of this one varies slightly from one sample to another, except for the spectrum of the irradiated sample TRME01, since it presents almost null absorption peaks or with very low intensity. In the case of the TRM10 sample, it presents absorption peaks of bands similar to the TRM22 sample.



Graph 7. Spectrum absorption from retromolar trigone region after gamma irradiation I the medium spectral infrared region

Conclusion

From the results, it was possible to establish a conclusion of the present study, emphasizing the direct effect of the ionizing radiation, in this case, the irradiation as a determinant and contributing factor to the loss of the physical, compositional and morphological properties of the hard tissues submitted to conventional radiotherapy, from an in vitro model, of patients affected by head and neck cancer, which in fact favors the appearance of undesirable effects of this therapeutic modality, independent of local or systemic contributing factors.

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