Effect of cumulative ionizing radiation on flexural strength, flexural modulus and elasticity modulus of dentin in unerupted human third molars

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Effect of cumulative ionizing radiation on flexural strength, flexural modulus and elasticity modulus of dentin in unerupted human third molars

Short Title: Mechanical properties of dentin after cumulative radiation therapy

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The authors report no conflicts of interest.

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Short Title: Mechanical properties of dentin after cumulative radiation therapy

KEY WORDS: dentin; elasticity modulus; flexural modulus; flexural strength; ionizing radiation; mechanical property; radiation; third molars; three-point bending test; wisdom teeth.

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Abstract

Purpose/Objective(s): This in-vitro study aimed to investigate the changes in mechanical properties in dentin of third molars after radiation therapy using variable doses and frequencies.

Materials/Methods: Rectangular cross sectioned dentin hemi sections (N=60, n=15 per group) (>7x4x1.2mm) were prepared using extracted third molars. After cleansing and storage in artificial saliva, random distribution was performed to 2 irradiation settings, namely AB or CD (A: 30 single doses of irradiation (2 Gray each) for 6 weeks, B: Control group of A, C: 3 single doses of irradiation (9 Gray each) and D: Control group of C). Various parameters (fracture strength/maximal force, flexural strength and elasticity modulus) were assessed using a universal Testing Machine (ZwickRoell). The effect of irradiation on dentin morphology was evaluated by histology, scanning electron microscopy, and immunohistochemistry. Statistical analysis was performed using two-way analysis of variance and paired and unpaired T-tests at a significance level of 5%.

Results: Significance could be found considering the maximal force applied to failure when the irradiated groups were compared to their control groups (A/B (P<0.0001) and C/D (P=0.008)). Flexural strength was significantly higher in the irradiated group A compared to control group B (P<0.001) and for the irradiated groups A and C (P=0.022) when compared to each other. Cumulative radiation with low irradiation doses (30 single doses; 2 Gray) and single irradiation with high doses (3 single doses; 9 Gray) make the tooth substance more prone to fracture, lowering the maximal force. The flexural strength decreases when cumulative irradiation is applied, but not after single irradiation. The Elasticity modulus showed no alteration after irradiation treatment.

Conclusion: Irradiation therapy affects the prospective adhesion of dentin and the bond strength of future restorations, potentially leading to an increased risk of tooth fracture and retention loss in dental reconstructions.
Introduction

Radiation therapy is a treatment for head and neck cancer that uses ionizing irradiation (1), and is commonly used as a primary adjuvant therapy to surgical treatment in conjunction with chemotherapy or as a palliative treatment for advanced or inoperable tumours (2). The oral cavity manifestations of gamma irradiation can include not only xerostomia, mucositis, candidiasis, dysgeusia, loss of taste, muscle trismus, vascular changes, and osteoradionecrosis, but also a possible contribution to an increased risk of irradiation tooth decay (3,4). Patients undergoing radiotherapy have an increased risk of developing irradiation caries throughout their life, not only during or immediately after treatment (5,6). Irradiation caries is caused by indirect and direct effects. Indirect effects include changes in salivary flow rate and saliva quality, difficulty in performing adequate oral hygiene, adoption of a soft diet due to difficult swallowing, and microbiota shift (6-8). Direct effects on the tooth include changes in the crystalline structure, dentinoenamel junction, acid solubility of enamel and microhardness caused by alteration in the organic matrix (2,5,6).

The enamel of non-irradiated teeth presents organized prisms with transverse and oblique arrangement surrounded by interprismatic portions. The prismatic structure remains unaltered after irradiation, while the interprismatic region becomes more evident (4). It has been reported that irradiation affects the organic matrix of enamel (9,10). Free radicals and reactive oxygen species that react with water in the interprismatic region are considered to contribute to the changes. The dentin of non-irradiated teeth presents well-defined dentinal tubules and an organized collagen fiber network. Morphological alterations manifest after 30 and 60 Gy irradiation doses in the intertubular, peritubular and intratubular dentin. At 30 Gy fissures in dentinal structure become evident, while at 60 Gy doses, the dentinal tubules become obliterated. The collagen fibers gradually fragment with the increase of irradiation doses (4). Free radicals affect the hydration of collagen fibers harmfully if the irradiation causes alteration in the secondary and
tertiary structures of proteins. Degradation of the collagen fibers network and obliteration and fissures in the dentinal structure are a result of loss of collagen fibers hydration (11). Increasing the dose results in progressive micromorphological alterations of both enamel and dentin structures. While the microhardness of the permanent teeth increases after irradiation, the values for the microhardness of the enamel in the superficial depth decrease up to a cumulative dose of 30 Gy, but increase at higher doses, with the middle and deep enamel do not differing from non-irradiated enamel. The superficial and deep dentin microhardness has no alteration compared with the non-irradiated dentin, while the middle dentin microhardness decreases significantly. Overall, the microhardness of dentin decreases after every 10 Gy cumulative dose from 10 Gy up to 60 Gy dose (4).

A possible explanation could be that dentin has a higher water content than enamel (10% versus 4% by weight). Hence, tissue with higher water content could be more vulnerable to the radiation effects and have stronger effects on mechanical properties of tissues (12). As dentin supports enamel, a softer dentin tissue becomes less efficient, allowing the occurrence of fractures and cracks in the enamel (13). The higher microhardness of the superficial layer of enamel turns it more friable and susceptible to crack formation, possibly contributing to dentinal hypersensitivity and favouring marginal infiltration of restorations (4). The degradation of the organic portion of dentin could also interfere with the adhesion of resinous restorative materials (13).

Several studies focused on the visualization and interpretation of consequences of radiation therapy on the macromorphological structure changes in human permanent teeth, but without performing mechanical properties measurements such as flexural strength, flexural modulus or elasticity parameters. It has been reported that mechanical properties of dental tissue change after radiation therapy and consequently can affect the outcome of restorative dental treatment of patients with head and neck cancer undergoing radiotherapy (4). Therefore, the objectives of this study were to investigate the effect of cumulative ionizing radiation on mechanical properties of the dentin and to show structural and morphological alterations. The null hypothesis tested was that radiation doses would not show significant differences on the mechanical properties of dentin.
Materials and Methods

Pre experimental Procedures

This in vitro study received ethical approval from the Ethics Committee of (name and location of Ethics Committee - blinded for review). The extracted human unerupted third molars used in this study were provided by the Clinic of Cranio-Maxillofacial and Oral Surgery of the University of (blinded for review).

Specimen preparation

One rooted maxillary and mandibular unerupted third molars (n=30) were selected, stored (in distilled water and thymol solution at 4°C to inhibit microbial growth) and used in three weeks period post-extraction. The apical third of the root was embedded in epoxy resin/ acrylic blocks using a conventional composite (FiltekTM Supreme XTE Flowable Composite, 3M ESPE, St. Paul, MN, USA) to stabilize the tooth for the cutting procedure.

Thereafter, the tooth crown was cut off 1 mm below the cementoenamel junction and dentin specimens with a rectangular cross section (>7x4x1.2 mm) were cut in mesiodistal direction into buccal and palatinal / lingual halves of the teeth using an electrical precision diamond wire saw with blade diameter of 0.17 mm and 30 μm roughness under constant water cooling (Well, Walter Ebner, Locle, Switzerland). After cutting, they were polished manually under water flow with 1200 grit silicon carbide paper (Streuers, Willich, Germany) until a flat surface was obtained. The thickness was verified using a digital micrometer (Mitutoyo, Kamagawa, Japan). Finally, they were washed in running water, dried with gauze, ultrasonically cleaned in water for five minutes and placed in 12-well acrylic cell culture plates filled with artificial saliva, which was prepared according to the chemical components (chemical compounds of artificial saliva stock solution (sodium bicarbonate 2.4 g; potassium chloride 1.7 g; magnesium chloride 0.1 g; calcium chloride 0.2 g; potassium thiocyanate 0.2 g; potassium dihydrogen phosphate 0.7 g; boric oxide 0.1 g; double distilled water 1000ml) and artificial saliva (sodium bicarbonate 1.62 %, 51.5 ml; stock solution 2.4 g/l, 198 ml; double distilled water 198 ml; natrosol HR 2.5 g; glycerin 85%, 50 g)). The tooth
sections were obtained in order to perform 3-point bending tests, SEM and immunohistochemical evaluation.

**Experimental design/ Radiation procedure**

Both hemisections of each tooth were randomly distributed to either the first two groups (A and B) or the last two groups (C and D). Groups A and C were irradiated with a cumulative dose of 60 Gy varying in sequences and single Doses. While Group A was irradiated with a dose of 2 Gy/fraction (1 fraction per day, 5 times a week) on a 6-weeks course, Group C was irradiated with 9 Gy/fraction (1 fraction per day, 3 times) (total dose = 60 Gy). The total dose was in both groups 60 Gy. Groups B and D served as non-irradiated control groups of Groups A and C. During the radiation process the specimens were stored in 1ml sodium chloride in the outer 16 wells of the 24-well plate, to minimize the radiation inaccuracy (Figure 1A) caused by scattering (measurements revealed less than 5% difference of the absolute dose calibration between the outer wells) as shown in Figure 1 B-C, showing the radiation set up. The radiation was carried out by a 220 V unit (Gulmay D3225/ GM 0196, Gulmay Medical LTD, Surrey, England) (Applicator dimension 20cm x 20cm, Tube current 15 mA, Dose 120 MU corresponds to 1 Gy at the 4 edges of the plate, no Gap between applicator and tissue culture plate. Between irradiation sessions the specimens were stored in an incubator (Binder GmbH, Tuttlingen, Germany) at 37°C in artificial saliva, which was renewed daily.

**Mechanical properties evaluation/ 3-point flexural strength**

The three dimensions (length, width and height) of each specimen were measured and tested in the Universal Testing Machine (ZwickRoell) using a metallic jig inducing the load at a speed of 1 mm/min to the center of the specimen surface until fracture. Tests were performed according to ISO 10477:1992 (14,15).

Thereafter, the flexural strength (σ in MPa) for the rectangular sample was calculated using the following formula (15): \( \sigma = \frac{3 \cdot F_{\text{max}} \cdot L}{2 \cdot b \cdot d^2} \), where \( F_{\text{max}} \) = maximum force (Newton) was applied for the
fracture; L=distance (in mm) between the lower supports (span), in this study a 7 mm span was used; b= width of specimen (4 mm); d=thickness of the specimen (1.2 mm).

Furthermore, the elasticity/ flexural modulus (E in MPa) was calculated using the following formula (15):
\[ E = \frac{F_{\text{max}} \cdot L^3}{4 \cdot w \cdot t^3 \cdot y} \]
where \( F_{\text{max}} \) = maximum force (Newton) applied for the fracture of the specimen; 
\( L \) = distance (in mm) between the lower supports (span), in this study a 7 mm span was used; 
\( w \) = width of specimen (4 mm); 
\( t \) = thickness of the specimen (1.2 mm); 
\( y \) = deflection at load point.

**Scanning Electron Microscopy (SEM)**

Hemisections of the same teeth were assigned to 3 groups (A: Ctrl, B: \( \text{H}_3\text{PO}_4 \) (37%, 1 min), C: EDTA (5 %, 1 min)). Specimens in group A were not further treated and stored in artificial saliva. Both hemisections (the irradiated and the non-irradiated) in group B were stored in 37% phosphoric acid (\( \text{H}_3\text{PO}_4 \)) for 1 min, while the ones in group C were stored in 5% EDTA for one minute. Afterwards they were rinsed with distilled water for removing the smear layer.

The preparation procedure of biological specimens for visualisation under Scanning Electron Microscope (SEM) (JSM-6060, JEOL, Tokyo, Japan) included chemical fixation in glutaraldehyde, followed by dehydration in ascending acetone series (50-70-80-90-96-100%) using different durations (2x15 min, 2x15 min, 2x15 min, 2x15 min, 3x20 min, 2x1 hour). After air-drying at room temperature for 24 hours in desiccator, they were mounted on aluminium stubs and gold/palladium sputter-coated for 10 nm (90 s, 45 mA; Balzers SCD 030, Balzers, Liechtenstein).

SEM images were obtained at 10 kV, x1000, x5000, x10000, x20000 and x50000 magnification (Zeiss Supra V50, Carl Zeiss, Oberkochen, Germany).

**Histological evaluation**

The specimens were dehydrated in ascending acetone series (70-80-90-96-100%), embedded in EPON, cut with the microtome set at 3 mm and then stained in PAS and toluidine blue (Schroeder 1969).

The tests were performed in a Leica DM-RBEA microscope (x1000) (Leica, Wetzlar, Germany) equipped with an image system (Q-500MCA; Leica, Wetzlar, Germany). Digital microscope images were made at increasing magnifications (x5, 10, 20 and 40).
Immunohistochemistry evaluation

Characterization of dentin tissue using rabbit COL1A2 antibodies was performed on histological sections. The specimens were fixed in buffered formaldehyde (4%) for one day, demineralized in EDTA (12.5%) for two weeks and embedded in paraffin. Afterwards, they were sectioned and immunohistochemically stained. Therefore, the specimens were incubated with polyclonal rabbit anti-Col I antiserum (Nordic Biosite AB, Täby, Sweden) at 1/100 dilution, overnight at 4°C. Specimens were counterstained with Haematoxylin staining.

Statistical Analysis

Statistical analyses of control and post-irradiated specimens were performed by using the Statistical Package for the Social Sciences (version 18.0, SPSS Inc, Chicago, IL, USA). Kolmogorov-Smirnov and Shapiro-Wilk/ Weibull tests were used to test data normal distribution. Two-way ANOVA test revealed the statistical significance between the 2 radiation groups, while Wilcoxon test was performed to determine significance between the control and irradiated specimens of each group. The tested variables were: Maximal force, flexural strength and elasticity modulus. P values smaller than 0.05, were considered to be statistically significant for all the comparisons.

Results

Mechanical properties analysis

Significance could be found considering the maximal force (Fmax) applied to break the specimens when the irradiated groups were compared to their control groups, while no significance could be found when both irradiated groups were compared to each other regarding Fmax (Table 1 A-B). The mean Fmax values for the control groups were 108.2 MPa, for group A 72.1 MPa, and for group C 75.2 MPa. Group A showed lower mean values than C yet, no significance between both groups was observed. Regardless of the radiation method, Fmax decreased significantly compared to control measurements. Flexural strength showed significant difference for the irradiated group A when compared to its control group B and for the irradiated groups A and C when compared to each other. When compared to its
control group, irradiated group C showed no significance to D. The mean value of flexural strength for the control groups was 236.3 MPa, while for group A it was 170.2 MPa and for group C 174.9 MPa. Group A showed lower mean values than C, significance was observed.

After radiation elasticity modulus differences showed no significance, neither when irradiated groups were compared to each other nor to their control groups. The elasticity modulus values for the control groups varied between 2.7 and 13.6 MPa (Mean: 7.4 MPa), while they varied between 3.6 and 12.9 MPa (Mean: 5.9 MPa) for group A and between 2.5 and 12.2 MPa (Mean: 7.4 MPa) for group C. Group A showed lower mean values than C, showing significant differences.

The mean and standard deviations of ΔFmax, ΔFlexural strength and ΔElasticity modulus values are presented in Table 1A. An overview of all parameters tested, and statistical analyses conducted can be found in Table 1C.

**Scanning Electron Microscopy (SEM) Findings**

SEM images indicated alteration in the tooth substance micromorphology after radiation whether with low and frequent doses or with high and less frequent doses. Irradiated specimens showed changes in observable number and distribution of dentin canals in contrast to their control specimens. The inner structural morphology of the dentine canals was affected by the radiation. Pulpal morphology alteration could also be observed (Figure 2 A-F).

Using pretreatment methods as EDTA and H₃PO₄ allowed the inspection of the fiber morphology by eliminating the debris resulting from the cutting procedure.

**Histological and Immunohistochemical Findings**

Digital images were made from specimen surfaces before and after radiation and staining or immunocytochemical treatment. Control specimens showed distribution of the number and canals per area, while treated specimens presented less and uneven distributed dentin canals. The antibodies showed a netlike even binding pattern. The irradiated specimens lost the binding pattern. Immunohistochemical images can be found in Figure 3 A-T and SEM images are illustrated in Figure 4 A-F.
Discussion

The present study was conducted to investigate the effect of cumulative ionizing radiation on mechanical properties of dentin and to show structural and morphological alterations in terms of fracture strength, flexural modulus and elasticity modulus in dentin after variable radiation doses and frequencies based on an in vitro study in extracted third molars. Considering the obtained results, the null hypothesis was partially accepted for the flexural outcomes and rejected for the elasticity properties.

Nowadays, head and neck cancer are the sixth most prevalent cancer with an approximate incidence of 600,000 cases a year in the world (16). Although various radiotherapy techniques have been introduced, modulated-intensity radiotherapy is currently the treatment of choice because it allows precise dosing of tumoral tissue and provides greater protection of adjacent healthy structures by applying doses ranging from 30 to 70 Gy, depending on tumor type and adjuvant tissue (17). Given this range, the study applied dose was 60 Gy. The ionizing radiation targets tumor cell death and operates through formation of free radicals of hydrogen and hydrogen peroxide, which can interact with water as an oxidizer and cause molecular denaturation (17-19).

Among others, two of the most common oral complications of radiation therapy are hyposalivation and xerostomia, which can affect the buffering and remineralization capacity of oral tissues. The reduced salivary flow may cause a change in the oral pH. Tooth enamel becomes prone to demineralization when the oral pH drops to 5.5 or less (20-22). The normal state of the enamel surface depends on the continuous demineralization and remineralization processes in the oral environment. When demineralization predominates, mineral losses and damages to hydroxyapatite and matrix decomposition can occur (20,23,24). In addition to the involvement of the salivary glands, it has been suggested that radiation can cause a change in the mechanical and surface properties of teeth, especially dentin, due to the high amount of water inside the structure, damaging the tissue by the changes in organic structures and collagen fibers (19,25,26). This study provides robust support for this theory, demonstrating that the dental structures in irradiated patients are compromised. The irradiation affected the mechanical,
biological and physical properties of dentin. In addition, the adhesive capacities can be damaged by the biological decomposition of the collagen fibrils (4,19,27). Considering the obtained results, the present study, confirmed and coincided with the previous literature, in which radiation was reported to cause alterations in dental tissues, directly affecting the mechanical and morphological characteristics. The mechanical properties evaluation showed that radiation causes reduction of dental tissues and impairment of mechanical properties, such as hardness, flexural strength and elasticity modulus.

Cumulative radiation decreases the amount of organic matrix of the enamel through the degradation of reactive oxygen species of the intertubular and intratubular structure. In addition, irradiation causes obliteration of the dentinal tubules, dehydration of collagen and alteration of secondary and tertiary structures of the proteins. Therefore, it can be hypothesized that radiation therapy should decrease flexural strength and flexural modulus of the tooth substances dentin and enamel.

The mechanical analysis showed a statistically significant variation in the Fmax values in the group of irradiated dentin compared to those in the control group. This is in line with other studies in which a reduction in the microhardness of the enamel and dentin regions has been observed when subjected to the cumulative radiation doses up to 60Gy (4,28). Several studies confirmed that changes on the teeth produced due the radiotherapy alter the mechanical properties of these tissues (29).

Likewise, the flexural strength showed significantly decreasing results for irradiated dentin compared to control groups. These data coincided with a previously published study by Franzel and colleagues, reporting a decrease in the hardness of enamel and dentin, along with a decrease in the elastic modulus of enamel and dentin following 60 Gy in-vitro irradiation (30). The elasticity modulus did not show any significant differences between irradiated and non-irradiated dentin, but with decreased modulus of elasticity in irradiated. These data are confirmed with other studies in which there was a reduction in the elastic modulus of enamel by 60% and 45% in dentin (30,31).

The decrease of these mechanical properties in the enamel could be related to changes in the interaction between the organic matrix and the apatite crystals and micro cracks formation in the hydroxyapatite minerals (29). These changes at the mechanical level are induced by the changes in their
structure and composition and can lead to fractures of the teeth. In addition, radiation could affect the teeth proprioception in humans and influence biting forces, which together with the weakening of the teeth would be another risk factor for fractures.

Considering the dental surface findings using histological and immunohistochemical analyzes, alterations of the micromorphology of dental surfaces and in the antibodies could be observed. Radiated specimens showed changes in the observable number and distribution of dentin canals in contrast to their control specimens. A massive demineralization of the teeth, especially in dentin could be observed after radiation therapy. In some other studies, it was reported that signs of destruction of the prismatic structure and remineralization of the damaged tissue were evident (29).

Based on the findings, there was a decrease in the organic matrix of the enamel and the reactive oxygen species degrade the tubular structure, obliterate the dentinal tubules, dehydrate the collagen and alter the secondary and tertiary structures of the proteins (29). Clinically, this situation may lead to a decrease in flexural strength and the before mentioned modulus of tooth flexion.

One performed in-vitro study, where extracted third molars were irradiated with up to a cumulative 31.5 Gy dose during 5 days mentioned that no measurable destruction of collagen could be detected (32). This phenomenon was also observed in this study, as alterations with high few doses affected the collagen structures less compared to more frequent low doses. The limit of measurable matrix transformation and thereby significant poorer mechanical properties is still unknown and needs further investigation. The changes in the surface of the teeth observed in the anatomy of the dentinal tubules can affect the adhesive capacity of the teeth and affect the future hybrid layer which may compromise bonding strength of future restorations.

Observing the histological and immunohistochemical results, irradiated samples presented dentin channels distributed less evenly and loss of the binding pattern of the antibodies compared to the control group. Immunohistochemistry was performed with rabbit COL1A2 antibodies binding to type I collagen, a member of collagen group I (fibril-forming collagen). Type I collagen is responsible for formation the fibrils of tendon, ligaments and bones. In bones the fibrils are mineralized with calcium hydroxyapatite. The C-
terminal propeptide, also known as COLFI domain, has crucial roles in tissue growth and repair by controlling both the intracellular assembly of procollagen molecules and the extracellular assembly of collagen fibrils. It binds a calcium ion which is essential for its function (33). Considering these findings and the adhesion properties, the lack of inorganic content in the enamel could make it difficult to achieve a stronger adhesion capacity, while a higher organic content in dentin could make bonding more problematic (34).

Accordingly, to the present study, the changes on the network-like binding pattern could negatively influence the characteristics of the dental surface since it has been shown that dentine collagen fibrils contain inactive forms of MMP proteolytic enzymes (MMP-2, -3, -8, -9 and -20) that form in the physiological and pathological processes in dentin. Furthermore, the most important negative factor affecting the resin-dentin bond has been reported to be the incomplete infiltration of the resin into the acid-etched dentin surfaces and deterioration of the interfacial bonding of the resin-dentin interface (35). The degradation of the resin-dentin bond caused by radiation could be complicated by the absence of the collagen fibrils necessary in the hybrid layer after the application of total or self-etch acid etch systems (36-38) causing catastrophic failures. The use of protease (MMP) inhibitors, such as chlorhexidine (CHX) is advised in case of bonding procedures of resin composite or partial and full crowns in irradiated people, as it was demonstrated that it would prevent the collagenous breakdown at the hybrid layer (39-42).

Future in-vitro studies should consider the simulation of the xerostomia experienced by patients during radiation therapy by reducing the saliva storage time and daily application of neutral sodium fluoride, which is applied in splints during radiation therapy, to reduce the side effects. However, the extent of the prevention and treatment possibilities through dental rehabilitation of irradiated humans needs further animal studies and clinical investigations with a focus on all dental hard tissues, such as enamel, dentin and also the pulp to simulate the in-vivo situation, as the extracted teeth specimens do not receive a nutritional biology supply compared to the in-vivo scenario.

When limitations are considered, the study indicated that radiation treatment using cumulative frequent low doses alters the anatomy of the dentin tubules by reactive oxygen species degradation of the tubular
structure, obliteration the dentinal tubules and dehydration of the collagen. A decrease of flexural strength of dentin compared to single high doses is more frequent. The elasticity modulus of dentin showed no alteration after radiation treatment. The changes in the surface of the teeth observed in the anatomy of the dentinal tubules can affect the adhesive capacity of the teeth and affect the future hybrid layer which may compromise bonding strength of future restorations, in case of the use of resin composite, amalgam, glass ionomer cements and resin modified glass ionomer cements as restoration materials.

Considering the clinical relevance in dental rehabilitation of patients with a history of radiation therapy of the oral cavity, clinicians should be aware of the increased risk of tooth fracture and retention loss of fillings and reconstructions.
References


Legends to figures and tables:

Figures:

**Figure 1:** A-C) A Radiation setting shown in 24-well acrylic cell culture plate 120MU corresponds to 1 Gy at position 1, 2, 3, and 4 of the tissue testplate. B Scheme and C measurements of radiation dose for each well, filled with 1ml Natrium chloride to test dose calibration.

**Figure 2:** A-F) SEM images of outer (peripheral) A B and C and internal (pulpal) D, E and F specimen side for A, D Control specimen, B, E irradiated (30 times with 2 Gy) and C, F irradiated (3 times with 9 Gy) at 3 different magnifications 1000x, 5000x, 20000x.

**Figures 3:** A-T) Digital microscope images of immunohistochemically prepared and non-treated/ control specimen A, K at the magnifications B, L) 5x, C), M) 10x, D), N) 20x and E), O) 40x vs. a irradiated specimen (30 times by 2 Gray dose) F) at the magnifications G) 5x, H) 10x, I) 20x and J) 40x vs. a irradiated specimen (3 times by 9 Gray dose) P) at the magnifications Q) 5x, R) 10x, S) 20x and T) 40x.

Please Note that the specimens A to J and K to T belong to the same two teeth. (Arrow: less and uneven distributed dentin canals in irradiated specimens).

**Figures 4:** A-F) SEM images of A) Control (non-irradiated), B) irradiated, C) Control (non-irradiated), H₃PO₄ treated, D) Irradiated (30 times with 2 Gy), H₃PO₄ treated, E) Control (non-irradiated), EDTA treated, F) Irradiated (3 times with 9 Gy), EDTA treated specimens at 5 different magnifications, namely x1000, x5000, x10000, x20000 and x50000

**Figure 5:** A-C) The Weibull graph and moduli for all experimental groups A, B, C and D for the parameters A) ΔMaximal Force (ΔFmax, N), B) ΔFlexural strength (ΔFS, MPA) and C) ΔElasticity modulus (ΔEM, MPA).

Tables:
Table 1: A-B) Cross-comparison of significant differences between $\Delta$Maximal Force ($\Delta F_{\text{max}}$), $\Delta$Flexural strength ($\Delta F_S$) and $\Delta$Elasticity modulus for groups A, B, C and D based on the test method (Two-way ANOVA test, Wilcoxon test $\alpha = 0.05$). For group descriptions see Fig 4. B The mean and standard deviations of $\Delta$Maximal Force ($\Delta F_{\text{max}}$, N), $\Delta$Flexural strength ($\Delta F_S$, MPA) and $\Delta$Elasticity modulus ($\Delta E_M$, MPA) values for the groups A and C compared to their control groups B and D, Weibull modulus per experimental group analyzed after 3-bending fracture tests.
Figure 1 A Radiation settings shown in 24-well acrylic cell culture plate 120MU corresponds to 1 Gy at position 1, 2, 3 and 4 of the tissue testplate. B Scheme and C measurements of radiation dose for each well, filled with 1ml Natrium chloride to test dose calibration.

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Figure 2. A-F SEM images of outer (peripheral) A, B, and C and internal (pulpal) D, E, and F specimen side for: A, D Control specimen, B, E irradiated (30 times with 2 Gy) and C, F irradiated (3 times with 9 Gy) at 3 different magnifications 1000x, 5000x, 20000x.
Figure 3

A)  
B) 
C)  
D)  
E) 
F)  
G) 
H) 
I)  
J)
Figure 3 a-t) Digital microscope images of immunohistochemically prepared and non-treated/ control specimen A), K) at the magnifications B), L) 5x, C), M) 10x, D), N) 20x and E), O) 40x vs. a irradiated specimen (30 times by 2 Gray dose) F) at the magnifications G) 5x, H) 10x, I) 20x and J) 40x vs. a irradiated specimen (3 times by 9 Gray dose) P) at the magnifications Q) 5x, R) 10x, S) 20x and T) 40x. Please Note that the specimens A to J and K to T belong to the same two teeth. (Arrow: less and uneven distributed dentin canals in irradiated specimens).
Figure 4 A-F) SEM images of A) Control (non-irradiated), B) irradiated, C) Control (non-irradiated), H$_3$PO$_4$ treated, D) Irradiated (30 times with 2 Gy), H$_3$PO$_4$ treated, E) Control (non-irradiated), EDTA treated, F) Irradiated (3 times with 9 Gy), EDTA treated specimens at 5 different magnifications, namely x1000, x5000, x10000, x20000 and x50000

Figure 5

Figure 5: A-C) The Weibull graph and moduli for all experimental groups A, B, C and D for the parameters A) ΔMaximal Force (ΔFmax, N), B) ΔFlexural strength (ΔFS, MPA) and C) ΔElasticity modulus (ΔEM, MPA)
Table 1

A Cross-comparison of significant differences between ΔMaximal Force (ΔFmax), ΔFlexural strength (ΔFS) and ΔElasticity modulus for groups A, B, C and D based on the test method (Two-way ANOVA test, Wilcoxon test \( \alpha = 0.05 \)). For group descriptions see Fig 4. B The mean and standard deviations of ΔMaximal Force (ΔFmax, N), ΔFlexural strength (ΔFS, MPA) and ΔElasticity modulus (ΔEM, MPA) values for the groups A and C compared to their control groups B and D, Weibull modulus per experimental group analyzed after 3-bending fracture tests.

<table>
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<th>Group C/D</th>
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Table 1A-B.