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Microstructural changes in the irradiated and osteoradionecrotic bone: a SEM study

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ABSTRACT

Radiation exposure is a major health concern due to bone involvement including mandible, causing deleterious effects on bone metabolism, and healing with an increasing risk of infection and osteoradionecrosis. This study aims to investigate the radiotherapy-induced microstructural changes in the human mandible by scanning electron microscopy (SEM). Mandibular cortical bone biopsies were obtained from control, irradiated, and patients with osteoradionecrosis (ORN). Bone samples were prepared for light microscopy and SEM. The SEM images were analyzed for the number of osteons, number of Haversian canal (HC), diameter of osteon (D.O), the diameter of HC (D.HC), osteonal wall thickness (O.W.Th), number of osteocytes, and number of osteocytic dendrites. The number of osteons, D.O, D.HC, O.W.Th, the number of osteocytes, and osteocytic dendrites were significantly decreased in both irradiated and ORN compared to controls ($p < .05$). The number of HCs decreased in irradiated and ORN bone compared to the control group. However, this was statistically not significant. The deleterious effect of radiation continues gradually altering the bone quality, structure, cellularity, and vascularity in the long term (>5 years mean radiation biopsy interval). The underlying microscopic damage in bone increases its susceptibility and contributes further to radiation-induced bone changes or even ORN.

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Introduction

Radiotherapy (RT) plays an important role in the treatment of head and neck cancer (HNC) patients, and it is estimated that approximately 80% of HNC patients receive RT once during their disease course.[1] The main goal of RT is to restrict the tumor cells but along with tumor cells, some irreversible damage is inflicted on healthy tissues surrounding the tumor. Osteoradionecrosis (ORN) is a well-known complication of RT that represents a slow-healing radiation-induced ischemic necrosis of variable extent. The incidence of ORN ranged from 2% to 22% reflecting not only the difference in RT techniques but also the uncertainty in the definition and diagnosis of ORN.[2] However, in recent times, the ORN incidence has shown a decline to 4–8%, possibly resulting from the improvement in RT techniques like intensity-modulated radiation therapy (IMRT).[3,4]

Osteoradionecrosis is more common in the mandible than in the maxilla probably due to its compact structure and relatively poor vascularization. Several hypotheses are considered to explain the pathophysiological mechanism of ORN which included the "3Hs" (hypoxia, hypovascularization, and hypocellularity) and "2Is" (infection and ischemia).[5,6] Later, the "fibroatrophic theory" was proposed as the main mechanism of ORN.[7] It is suggested that the interaction of radiation with the tissue generates reactive oxygen species (ROS) causing endothelial cell injury associated with vascular thrombosis, necrosis, ischemia, and tissue loss.[8]

Approximately 80% of total body bone mass consists of cortical bone that provides the majority of the mechanical strength. Osteon, osteocytes, and lacuno-canalicular network (LCN) are part of the Haversian system that

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can sense and transduce mechanical stress in response to external stimuli.[9] Jawbones are often exposed to RT because of their proximity to the tumors. The shape and size of the osteon are important in resisting the deformation of the bone which are altered due to irradiation. Irradiation also causes a reduction in the bone matrix as it inhibits osteoblastic bone formation and modifies the bone channel network.[10] Delayed radiation-induced injury to bone has been attributed either to vascular damage or as a result of the combined effects of vascular and cellular changes.[11]

Microscopic analysis of irradiated bone has shown a decreased osteon number,[12] increased empty osteocytic lacunae,[13] decreased osteocytes,[11,14] an increased number of osteoclasts and osteoclastic activity,[15] changes in Haversian canal diameter, decreased and obstructed blood vessels,[14,16] increased fibrosis,[14] and increased percentage of adipocytes in bone marrow.[14,17] The effect of irradiation on bone is mostly investigated in animal long bone models.[10,11,13,15,16] Long bones differ in embryological origin and the ossification process compared to the jawbone.[18] Hence, this causes difficulty in transposing results to the jawbone. A recent Raman spectroscopy study[19] has shown that radiation could alter the biochemical composition of the human mandible thereby affecting the quality and mechanical competence of bone. This increases radiation-associated risks and could contribute to the pathophysiology of ORN. Scanning electron microscope (SEM) is an effective analytical tool in the assessment of bone microarchitecture in healthy individuals and diseased patients along with traditional light microscopy.[20]

As the adverse effects of irradiation are multifactorial and oral rehabilitation procedures using dental implants are frequently indicated in irradiated patients, it is mandatory to understand the underlying factors that influence and cause necrosis of the jawbone. We assume that RT would continuously change the morphology and cellular and vascular contents as a long-term change in irradiated bone. This study aims to analyze the histological and microstructural alterations in human cortical bone following RT in irradiated and ORN jawbone. We focused on the changes in mandibular cortical bone osteon, Haversian canals, osteocytes, and LCN of irradiated and ORN bone.

Materials and methods

Study design

Mandibular bone biopsy samples were obtained from 31 edentulous patients (23 males and 8 females) during the dental implant surgery and were categorized into three study groups. Group 1, control (C) consisted of clinically healthy patients, without a history of oral mucosal lesions, oral cancer and RT ($N = 11$), Group 2, irradiated (IR) included patients with a history of RT to treat HNC and no signs of any inflammation, ulceration or oral mucosal lesions at the time of biopsy ($N = 14$) and Group 3, osteoradionecrosis (ORN) ($N = 6$) patients with ORN who had visited the Department of Oral and Maxillofacial Surgery in Amsterdam UMC for surgical management of the osteonecrotic lesion in the mandible that did not respond to conservative treatment (i.e. long-term systemic antibiotics and/or topical application of antiseptic agents). All the IR patients had received an estimated dose of 50 Gy or higher on the anterior mandible and were treated with 20 sessions of hyperbaric oxygen therapy preoperatively and 10 sessions postoperatively (Marx protocol) as a standard procedure. To obtain sample homogeneity, the female subjects selected in the study groups were of post-menopausal age (over 50 years). All patients had normal blood calcium, phosphate, parathyroid hormone, and HbA1c levels. Patients with impaired bone metabolism (e.g. hyperparathyroidism, osteomalacia), bisphosphonate medication, or systemic immunosuppressive medication up to 3 months before sample harvesting were not included in the study. The demographics of the three patient groups, radiation dose details, and biopsy interval are presented in Table 1.

The study was approved by the Medical Ethical Committee of the Amsterdam University Medical Centers (location VU Medical Center, Amsterdam, the Netherlands (Registration number 2011/220)) and the Research Ethics Committee of the Northern Savo Hospital District (754/2018).

Table 1. Demographic data and distribution of the patient groups.

	Patients n = 31	Male/Female	Age range	Total dose	Local dose	Radiation- biopsy interval
Control group	11	7/4	43–74 yrs	-	-	-
Irradiated group	14	11/3	51–74 yrs	54–70 Gy	18–62 Gy	11–199 months
ORN group	6	5/1	51–84 yrs	56–70 Gy	56–66 Gy	13–90 months

ORN – Osteoradionecrosis.

Written informed consent was obtained from all individual participants included in the study.

Bone biopsy retrieval and specimen preparation

The patients in the control group were given a single dose of antibiotic prophylaxis (amoxicillin 3 g orally) 1 hour before the dental implant surgical procedure. The surgery was performed under local anesthesia by a single oral and maxillofacial surgeon at the Alrijne Hospital in Leiderdorp, the Netherlands. The irradiated patients were treated in the Department of Oral and Maxillofacial Surgery, Amsterdam University Medical Centers (Amsterdam UMC), location Vrije Universiteit Medical Center (VUmc), in Amsterdam, The Netherlands. The surgery was performed under general anesthesia, and patients were given antibiotic prophylaxis following the ORN-protocol (amoxicillin/clavulanic acid 500/125 mg three times daily) 24 hours before dental implant surgery and continuing 10 days post-surgery. ORN patients with extensive osteonecrotic lesions need surgical debridement. A sample of the excised tissue is sent to the Department of Pathology for routine examination to exclude the recurrence of malignancy within the lesion. The remaining tissue was used for the research.

The dental implant procedure was similar in both the control and irradiated group. A crestal incision was made in the interforaminal region of the mandible with a mid-line buccal release incision. A full-thickness mucoperiosteal flap was raised to expose the alveolar ridge and leveled by vertical alveolotomy if required. Implant preparation was made with a 3.5 mm diameter trephine drill (Straumann® Dental Implant System; Straumann Holding AG, Basel, Switzerland) to a depth of 10 mm at both canine regions, under continuous irrigation with sterile saline. The obtained bone specimen per patient was selected and prepared for further analysis.

The cortical bone specimens obtained from the mandible were fixed and dehydrated with increasing concentrations of ethanol and embedded in Poly-methyl methacrylate (PMMA: Merck KGaA). The cut surface of the PMMA bone block was used for microscopic analysis. For light microscopy (LM), thin sections (10 µm) were prepared and stained with Masson-Goldner trichrome. The slides were observed (10×) and photographed with a Zeiss AxioImager M2 (Carl Zeiss Microscopy GmbH, Jena, Germany).

Scanning electron microscopy (SEM)

For SEM, the bone blocks were acid-etched with 9% phosphoric acid for 30 seconds.[20] Later, the blocks were immersed in 5% sodium hypochlorite for 3 min after etching and rinsed in distilled water. The blocks were placed in a desiccator overnight and thereafter coated with a thin 30–40 nm gold layer for SEM imaging. The gold-coated specimens were examined and photographed using Zeiss Sigma HD VP (Carl Zeiss GmbH, Oberkochen, Germany) scanning electron microscope operated at 15 kV.

Histomorphometric analysis

From each bone sample, under 400× magnification, three Regions of Interest (ROI, 750 × 500 µm^2) with osteon were selected for SEM imaging to evaluate the osteons and Haversian canals (HC). Three small areas (100 × 100 µm^2), from each ROI (two osteonal areas and one interstitial bone area), were selected for further imaging at higher (2000×) magnification to evaluate the osteocyte number and dendrites (Figure 1). The following parameters were assessed: number of osteons, number of HC, the diameter of osteon (D.O), diameter of HC (D.HC), osteonal wall thickness (O.W.Th), number of osteocytes/100 ×100 µm,^{2} and number of osteocytic dendrites. Three investigators (PSR, BK, KV) were blinded

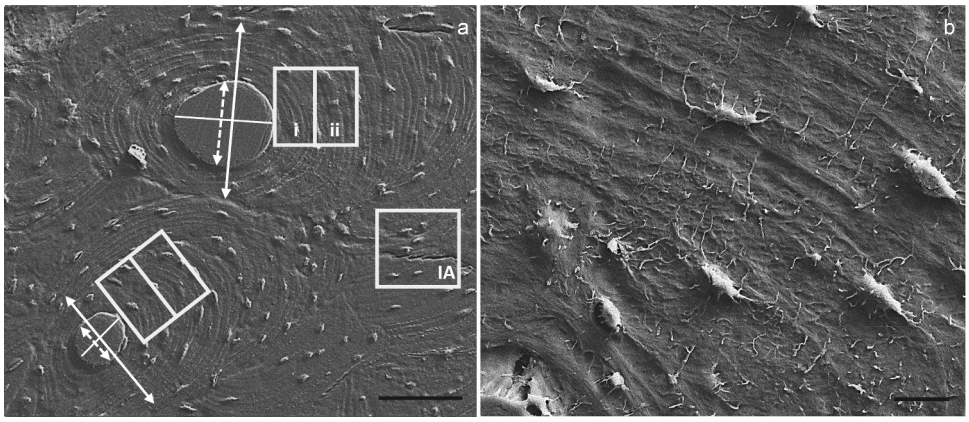

Figure 1. Scanning electron microscopic images showing histomorphometric measurements. (a) The measurement of an osteon, Haversian canal (HC) diameter, and the two osteonal (i – near to HC, ii – far to HC) and one interstitial area (IA) selection in the shown region of interest (ROI) (400× magnification; bar = 100 µm). (b) The number of osteocytes and their dendrites are appreciated in the osteonal area for evaluation (100 ×100 µm^2; 2000× magnification; bar = 10 µm).

for the assessment of histomorphometric parameters from the SEM digital images.

From ROI images with 400× magnification, the number of osteons and Haversian canals were calculated, and the diameters were measured using Carl Zeiss SmartTiff V3 imaging software. The diameter was measured as the shortest perpendicular distance to the longest axis of the osteon and Haversian canal in all the ROIs. The osteons with well-defined or almost complete cement-lined boundaries were considered in the count. The osteonal wall thickness was calculated for each osteon as half of the difference between the osteonal diameter and Haversian canal diameter (D.O – D.HC)/2 as suggested previously.[21]

From small area images with 2000× magnification, the number of osteocytes/100 × 100 µm^2 and their dendrites were manually counted in each selected osteonal and interstitial region. The area without any Haversian canal and the osteonal structure was considered an interstitial area for counting osteocytes. Further, in the osteonal area, the number of osteocytes and their dendrites were calculated by subdividing into near and far Haversian canal areas (osteocytes/100 × 50 µm^2).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS v 27.0, IBM). The Shapiro–Wilk test was performed to check the normality of the data. Non-parametric Kruskal–Wallis one-way ANOVA test with pairwise comparison was performed to determine the differences between the control, irradiated, and ORN groups. The differences were considered statistically significant at $p < .05$ level.

Results

Qualitative analysis under light microscopy showed more empty osteocyte lacunae in irradiated and ORN bone tissues. Haversian canals were surrounded with osteoid in the control, irradiated, and the ORN tissues, but the lamellar pattern was reduced in the ORN sample. The hypocellularity and inflammatory changes in the HC components were more evident in the irradiated and ORN samples (Figure 2).

The measurements obtained with SEM at 400× magnification are decreased in the ORN group compared to controls ($p < .05$); however, the number of HCs did not show any significant differences (Figure 3). Even though the parameters were decreased from control to irradiated groups, only the number of osteons showed significant differences. No significant differences were observed between irradiated and ORN bone tissue samples in any of the morphometric parameters measured at 400×. Microcracks were also evident more in the irradiated and ORN than in the control samples. The interstitial area showed more microdamage than the osteonal area of the bone samples.

The osteocytes and osteocytic dendrites were counted at 2000× magnification. The count was

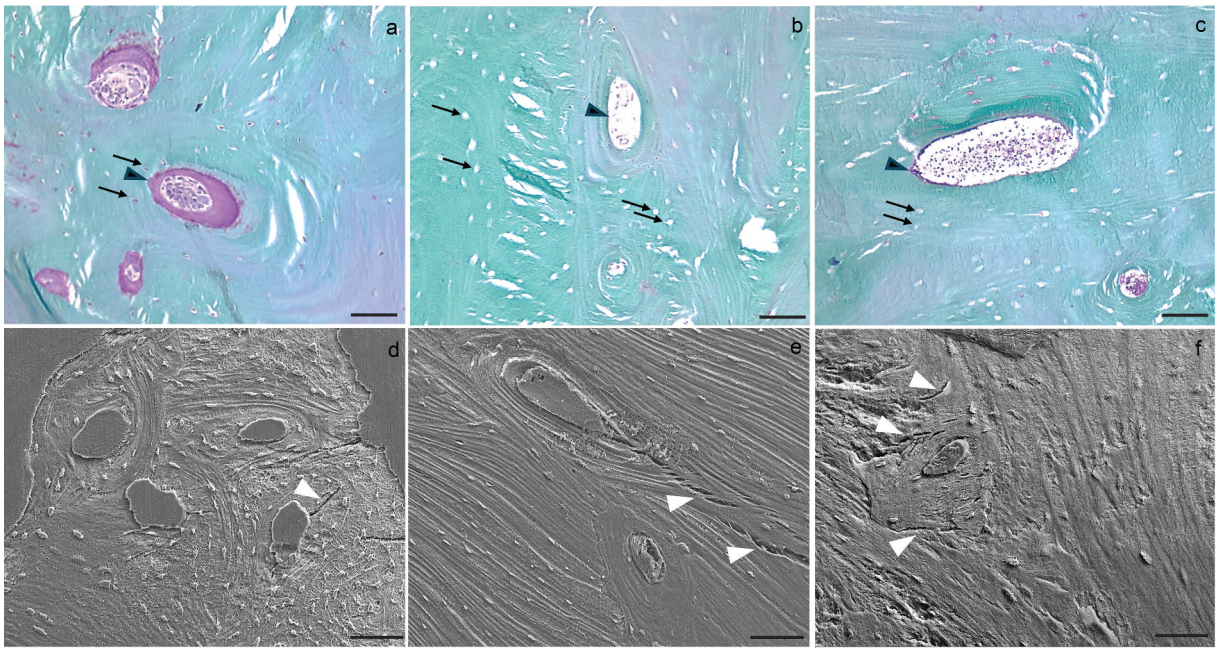

Figure 2. (a) In controls, HCs with their components (black arrowhead), and osteocytes (dark purple stained) within osteocytic lacunae (arrows) are well observed. (d) The SEM image of the control shows osteocytes in osteonal and interstitial areas; few microcracks are visible (white arrowheads). (b) The irradiated samples showed many empty osteocytic lacunae (arrows), few osteons, and hypocellular changes in the HCs (black arrowhead). (e) The SEM image shows few numbers of osteocytes and microcracks (white arrowheads). (c) In ORN samples, inflammatory cell infiltrates in the HC (black arrowhead), empty lacunae (arrows), and very few osteocytes are visible. (f) The SEM image shows very few numbers of osteocytes and an irregular osteon. Microcracks were observed (white arrowhead) (bar = 100 µm).

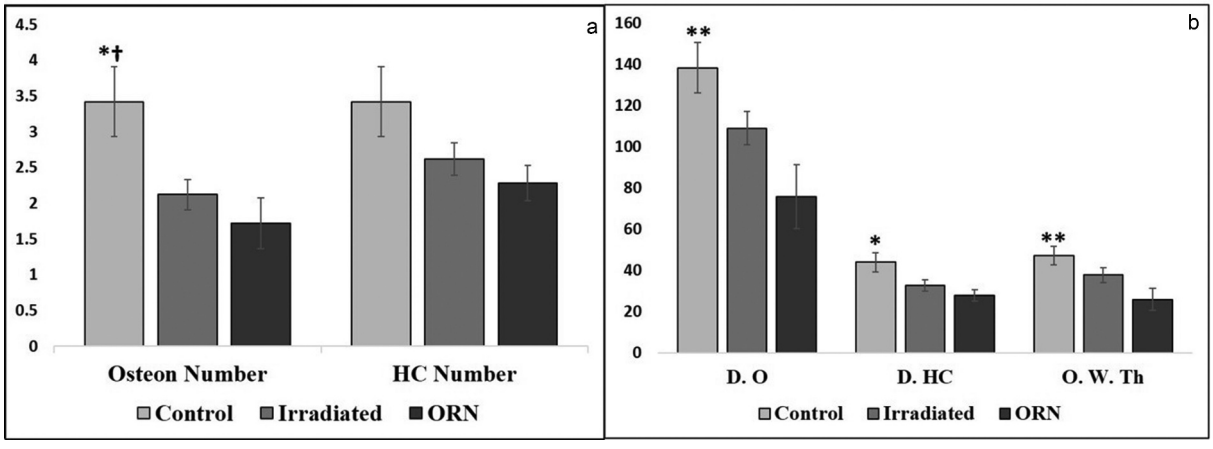

Figure 3. Comparison of histomorphometric parameters. (a) Osteon number, Haversian canal (HC) number in the regions of interest (ROI) (750 × 500 µm^2). (b) Diameter of the osteon (D.O), diameter of Haversian canal (D.HC), and osteon wall thickness (O.W.Th) between control, irradiated, and osteoradionecrosis (ORN) samples. **$p < .01$, *$p < .05$, *between control and ORN, † between control and irradiated.

decreased from controls to irradiated and further to ORN and was statistically significant (Table 2). The osteocytes and their dendrites in the osteonal area of controls showed a significant decline from the interstitial area when compared with irradiated and ORN ($p < .01$). When the osteocytes and their dendrites were observed near to HCs and far from HCs and compared, the decrease was statistically significant ($p < .001$) in irradiated and ORN than in the control group. The differences in the osteocytes and osteocytic dendrites in the osteonal and interstitial area did not show significant differences between irradiated and ORN.

Table 2. Histomorphometric parameters among control, irradiated, and osteoradionecrosis (ORN) studied with a scanning electron microscope (2000×).

Parameters	Control ($N = 11$) Mean ± SD	Irradiated ($N = 14$) Mean ± SD	ORN ($N = 6$) Mean ± SD
Total No. of Osteocytes/100 × 100 μm2	10.42 ± 2.73***††	7.05 ± 1.40	5.47 ± 2.13
a) Osteonal Area	13.91 ± 3.68***†††	8.33 ± 1.65	7.06 ± 3.10
i) Near HC	8.06 ± 2.25***†††	4.33 ± 1.33	3.44 ± 1.52
ii) Far HC	5.85 ± 2.00*†	4.0 ± 0.95	3.61 ± 1.68
b) Interstitial Area	6.94 ± 1.95**	5.76 ± 1.57‡	3.89 ± 1.71
Total No. of Osteocytic Dendrites	58.05 ± 31.13***†††	19.29 ± 8.76	16.72 ± 13.12
a) Osteonal Area	77.91 ± 45.72***†††	23.26 ± 11.37	19.00 ± 9.44
i) Near HC	46.03 ± 23.68***†††	11.19 ± 5.61	10.44 ± 5.47
ii) Far HC	31.88 ± 23.42***††	12.07 ± 7.28	8.56 ± 4.43
b) Interstitial Area	38.18 ± 17.95**††	15.31 ± 8.46	14.44 ± 17.32

HC = Haversian canal. ***$p < .001$, **$p < .01$, *$p < .05$, *between Control and ORN, †between Control and Irradiated, ‡between Irradiated and ORN.

Discussion

The present study demonstrates the long-term microstructural changes, bone structure, cellularity, and vascularity in irradiated and ORN mandibular bone. Osteocytic and dendritic changes due to irradiation are more significant than the osteon and HC morphology changes. Human mandibular bone is at risk of radiation injury due to its superficial location, compact structure, and paucity in vascular supply from inferior alveolar vessels and periosteum.[18]

The osteon structure performs a crucial role in bone mechanics and bone turnover. The heterogeneity of the osteon morphology following irradiation has been reported in an animal study.[22] The difference in the osteon sizes and morphology was evident in our study groups. The lamellar organization around the HCs was evident in our control and many irradiated samples both under LM and SEM. However, proper lamellar or round layered patterns were not present in many ROIs of our ORN samples. Similarly, osteon diameter and the osteonal wall thickness were consistently lower in irradiated and ORN bone. In some ORN samples, ROIs did not show any osteonal area. The decrease in osteon density in ORN samples was shown in a previous human study.[12] It is also reported that with advancing age, the osteon size decreases, and the proportion of extra-Haversian bone increases.[9,23] The alteration in the osteon morphology observed in our study could relate to irradiation, as the bone biopsy samples were retrieved from months to years after irradiation (>5 years), presenting as delayed changes. The decrease in osteon size following irradiation is reflected by the inhibited function and viability of osteoclasts, osteoblasts, and osteocytes, which influence osteon resorption and refilling with morphological alterations.[22,24] Hence, the slow and delayed bone remodeling might complicate bone healing, even with minimal dentoalveolar trauma, and can limit the success of dental implants in the long term.

The HC is a source of nutrients and sensation to osteocytes that reside within the lamellae and can initiate cortical bone remodeling. The HCs are adaptive to osteocytes as they are positively correlated to the osteon area and the osteocyte number.[24] In animal studies,[16,18,22,25] it has been revealed that irradiation causes changes in the HCs. A progressive decrease in the number of smaller vessels,[26] Haversian vessels, increased occlusion, and a decrease in cellularity have been reported as the long-term change following radiotherapy.[11] Under LM, the HCs containing blood vessels and supporting cells were clear in our control bone samples, whereas subsequent replacement of HC components with hypocellular changes was detected in the irradiated bone samples. The ORN samples showed necrotic tissue debris and inflammatory cell infiltrates in the HCs. The number and the diameter of the HCs as measured with SEM showed a consistent decrease in the study groups. This indicates that irradiation affected HC components and impaired the HC formation causing hypovascularity which may progress later to a delayed ischemic condition. The number of osteocytes decreases continuously, markedly affecting bone formation because of ischemia.

The osteocyte viability in irradiated bone is questionable as on one side irradiation causes damage to the osteoprogenitor cells and on the other hand, the osteocytes are radioresistant that remain viable for

several months but with compromised function.[27] Osteocytes account for 90% of all bone cells, and each osteocyte consists of around 50–100 dendrites that communicate and form a complex signaling network.[9] We observed dark purple stained osteocyte nuclei within the osteocytic lacunae and very few empty lacunae in the osteonal and interstitial bony areas of control samples under LM. The increase in empty lacunae and the presence of few osteocytic nuclei were observed more in our irradiated and ORN samples. These findings were confirmed with SEM, where the osteocyte numbers and their dendrites were considerably decreased. This results in affecting the bone lacuno-canalicular system, which provides an ideal environment for normal bone homeostasis through communication, transfer of exogenous and endogenous signaling pathways, the release of secondary messengers, transcription factors, and gene expression.[15,28–30] Empty osteocytic lacunae confirm osteocyte death due to reduced vascularity in our irradiated and ORN samples. A decreased osteocyte number and increased empty lacunae after irradiation were reported as the hallmark of the ORN.[12] In a recent study[17] on irradiated human mandibular cancellous bone, it was shown that irradiation causes early death of osteocytes, persistent suppression of osteoclastogenesis, and scarcity of osteoclast precursors, leading to the persistence of fragile bone matrix void of osteocytes.

Another observation in this study was the presence of microcracks in irradiated and ORN bone samples which might have been triggered due to stress concentrations near the osteons and the interstitial bone area. The presence of more microcracks in the interstitial area compared to the osteonal area was shown earlier and reported that the microdamage increases with the age of the bone.[31] Microcracks can result in the breaking of osteocyte dendrites, which subsequently results in osteocyte death.[32] Though we did not study the microcrack pattern, we assume that the empty lacunae would have been hypermineralized over time contributing to the bone brittleness and predisposing to microcracks under stress. The average number of dendrites in each osteocytic lacunae is decreased due to irradiation, which further decelerates the fluid flow and obstructs the signal transduction between osteocytes. It can negatively affect the mechanosensation system, resulting in impaired bone repair.[33]

The long-term sequential radiation-induced changes observed are hypocellularity, hypovascularity, increase of adipocytes in the marrow cavity, and focal areas of fibrosis in irradiated samples. Despite these underlying bony microscopic changes, all our irradiated patients had clinically normal oral mucosa at the time of biopsy. However, the changes due to irradiation in the overlying oral mucosa were previously reported suggesting changes in the oral epithelial cells by altering their surface morphology and prone to oral mucosal infection.[34,35] The administration of the HBO therapy to prevent ORN, in our patients might have slowed the radiation-induced damage over the years. It might have promoted the vascularity and survival of some of the osteocytes, as observed in our irradiated and ORN samples. Conflicting evidence exists regarding the effectiveness of HBO in the prevention of ORN, even though some studies have reported a lower incidence of ORN.[36–38] Also, we believe that the use of IMRT may contribute to a lower incidence of ORN cases, as suggested by other authors.[4,39]

Very limited studies on human jawbone are available investigating the quantitative parameters with SEM. In many animal studies, the radiation effects observed from a few days to a few months were reported as immediate and delayed changes. Our study shows the delayed radiation-induced changes that persisted over the years and affected the vulnerability of bone. However, this study has some limitations. First, the patient data on oral hygiene conditions, smoking habits, and alcohol consumption were not considered which may contribute to ORN development. Second, a low number of ORN patients were studied. Third, using a morphometric approach to study cortical bone leads to issues regarding tissue sampling differences and several technical factors affecting the measured parameters.

In conclusion, the findings observed in the mandible have confirmed that irradiation leads to long-term changes presented as decreased osteons, HCs, osteocytes, and LCN. Irradiated bone shows decreased remodeling and repair making it more susceptible to infection, which will contribute to the pathogenesis of ORN. Further, research with large sample sizes aiming to study sequential changes and recover bone quality under irradiated conditions may improve the clinical outcome in patients treated for head and neck cancers with radiotherapy.

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