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ORIGINAL ARTICLE

Dose-dependent effect of radiation on resorbable blast material titanium implants: an experimental study in rabbits

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ABSTRACT

Background: Radiotherapy is a commonly used treatment modality in head and neck cancer; however, it also negatively affects healthy structures. Direct damage to oral soft and hard tissue frequently occurs with radiotherapy. In this study, we aimed to evaluate the effect of radiotherapy on bone surrounding titanium dental implants via biomechanical and molecular methods.

Materials and methods: Fifty-four implants were inserted in the left tibiae of 18 adult male New Zealand rabbits (3 implants in each rabbit). After 4 weeks of the implant surgery, the left tibiae of 12 rabbits were subjected to a single dose of irradiation (15 Gy or 30 Gy). Four weeks after the irradiation, rabbits were sacrificed and removal torque test was done for the biomechanical evaluation. Bone morphogenetic protein-2 (Bmp-2) and fibroblast growth factor-2 (Fgf-2) expression analyses were performed with Real-time PCR. Statistical analysis was done using SPSS.

Results: The control group showed significantly higher removal torque value than the 15 and 30 Gy irradiation groups, and the 15 Gy irradiation group had higher removal torque value than the 30 Gy irradiation group ($p < .001$). The 15 Gy and 30 Gy irradiation groups had significantly lower Bmp-2 and Fgf-2 mRNA expressions than the control group ($p < .001$). In addition, the 30 Gy irradiation group had significantly lower Bmp-2 ($p < .01$) and Fgf-2 mRNA expressions ($p < .001$) than the 15 Gy group.

Conclusion: Radiotherapy with 15 and 30 Gy doses can adversely affect osseointegration of implants by reducing the quality of bone and impairing the bone-to-implant contact. The mechanism of action seems to be related to alterations in Bmp-2 and Fgf-2 mRNA expressions.

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Radiation; implant; dental; experimental study

Introduction

Dental implants are widely used for oral rehabilitation. Osseointegration is the main determinant for achieving implant stability and depends on many factors, such as the bone mineral density, volume and vascularity, and implant design and surface characteristics [1–5]. However, many factors may affect the osseointegration of an implant.

Radiotherapy for treatment of cancers of the head and neck region can adversely affect the healthy tissues in the oral cavity. It can also cause mild osteopenia, varying degrees of bone sclerosis, osteoradionecrosis and bone fracture following minimal trauma [6,7]. In addition, radiotherapy reduces the proliferation of bone marrow, and of periosteal and endothelial cells. As a result, the reduced viability of irradiated bone may prevent bone remodeling, thus the osseointegration and the success of implants could be impaired [8]. This process can result in failure of the dental implants. Implant stability, which is a prerequisite for treatment success, is determined by the quantity, quality and degree of bone-to-implant contact. The most commonly used biomechanical technique to assess this contact is the removal torque test. A positive correlation between the degree of bone-to-implant contact and removal torque value was reported [9].

For evaluation of bone quality, and bone formation and regeneration processes, several matrix proteins, growth factors, cytokines and chemokines can be used [10]. Bone is considered a reservoir for a variety of growth factors that are released during bone healing to stimulate recruitment and differentiation of mesenchymal stem cells [11,12]. Among these important growth factors, bone morphogenetic proteins (BMPs), vascular endothelial growth factor and fibroblast growth factor 2 (Fgf-2) play pivotal roles on the process of osteogenesis [13,14].

BMPs belong to a group of growth factors called transforming growth factors. Bmp-2 is a member of this family and has inducing and enhancing capacity on bone growth, and is regarded as the most potent growth factor on bone regeneration process [15]. It also promotes cell chemotaxis, proliferation and differentiation towards the osteogenic pathway [16].

Fgf-2 is one of the important molecules in this process. This growth factor can increase during acute wound healing

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and has a role on granulation tissue formation and tissue remodeling [10,17]. It also acts on the differentiation of osteoprogenitor cells to increase bone formation, and it stimulates periodontal ligament cell proliferation and migration; thus, Fgf-2 could be an attractive candidate for periodontal regeneration therapy [10,18].

Despite the side effects and negative influence on the dental implants, radiotherapy has been increasingly used in oral cancer patients over the past decade [19–21]. In addition, the extent of the impact is dependent upon the radiation dose. In this study, we aimed to evaluate the effects of 15 and 30 Gy irradiation doses on implants and its surrounding bone in a rabbit model, using biomechanical and molecular assays.

Materials and methods

Animal care

The animals were housed in facilities that follow international guidelines, and the experiments were approved by and conducted in accordance with the Institutional Animal Care and Use committee of Ataturk University. For this study, 18 adult male New Zealand white rabbits were achieved from the Ataturk University Experimental Animal Laboratory (ATADEM). During the study, the animals were kept in metal cages at a temperature of 22–24 °C and with a 12-h light/dark cycle; they were provided with standard commercial rabbit food and tap water *ad libitum*. The animals were randomly assigned into: 1. Implant control group (Group I, $n = 6$); 2. Implant +15 Gy irradiation group (Group II, $n = 6$); and 3. Implant +30 Gy irradiation group (Group III, $n = 6$).

Implant surgery

For the implant surgery procedure, the surgical region was shaved and disinfected. The animals were anesthetized with intramuscular injection of ketamine hydrochloride (Ketalar®, Eczacibasi, Turkey) at a dose of 50 mg/kg and xylazine hydrochloride (Rompun®, Bayer, Turkey) at a dose of 5 mg/kg. Fifty-four conical grade 5 titanium dental implants, 6 mm long and 2.8 mm diameter, were used in the present study (IMPLANCE, Trabzon, Turkey). A conical cavity (6 mm deep and 2.5 mm wide) was created in the lateral side of the tibia in a stepwise fashion, using color-coded, 6 mm-length surgical drills (2.0–2.5 mm diameter; IMPLANCE, Trabzon, TURKEY). The cavities were thoroughly rinsed with isotonic saline to remove bone fragments prior to the insertion of the titanium implants. Three implants were inserted in each animal. Implants were placed in the tibia of the rabbits following a standardized drilling procedure. Postoperative antibiotic and analgesic medications were administered.

Electron beam irradiation

After 4 weeks of implant surgery, the left tibia of each rabbit was subjected to a single dose of irradiation at 15 or 30 Gy dose. The high power electron beam linear accelerator (Siemens Primus LINAC) from the Department of Radiation Oncology at Ataturk University Hospital was used. Prior to irradiation, animals were anesthetized. A single dose of irradiation was delivered with a source–skin distance of 100 cm and field size of $25 \times 25\ cm^2$ with direct electron beam of 12 MeV electron, whereas the control leg was protected from radiation. The rabbits were sacrificed after 4 weeks following the irradiation.

Removal torque test

The removal torque test was used to evaluate the stability of the implants. The biological specimens were processed immediately after removal of the tibiae. After the stabilization of the tibia, the removal torque values were measured via a digital torque meter (Lutron TQ-880, Taiwan). A total of 18 implants (6 from each group) were retrieved. Measurements of peak torque to initiate reverse rotation were recorded in N-cm by a single blind examiner, and the mean torque values were calculated for each group [22].

Molecular investigations

Total RNA extraction and cDNA synthesis

Total RNA extraction and cDNA synthesis were performed according to the procedure described by Karakus et al. [23]. Briefly, the tissues (20 mg) were stabilized in RNA stabilization reagent (RNAlater, Qiagen), and then disrupted using the Tissue Lyser II (2×5 minutes for stomachs). Total RNA was purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, in a QIAqube (Qiagen, Hilden, Germany). The RNA samples were reverse-transcribed into complementary DNA by a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). Then, 10 µL total RNA were treated with 2 µL 10× RT Buffer, 0.8 µL 25× dNTPs mix, 2 µL 10× RT Random Primers, 1 µL MultiScribe Reverse Transcriptase and 4.2 µl DEPC-H_2O. Reverse transcription was carried out at 25 °C for 10 min, followed by 37 °C for 120 min, and finally, 85 °C for 5 min, using a Veriti 96-well thermal cycler (Applied Biosystems). cDNA concentration and quality were assessed using an Epoch spectrophotometer system and Take3 plate (Biotek, Highland Park).

Relative quantification of gene expression

Relative Bmp-2 and Fgf-2 expression analyses were performed with StepOne Plus Real-time PCR System technology (Applied Biosystems) using cDNA synthesized from rabbit tibiae RNA. qPCR was run using TaqMan Probe mix, TaqMan Probe-based technology (Applied Biosystems). Real-time PCR was performed using primers generated for rabbits: Bmp-2 Oc03824113_s1 Oc, Fgf-2 Oc03396228_m1 and β-actin Oc03824857_g1. For each sample, triplicate determinations were performed in a 96-well optical plate for both targets using 9 µL of cDNA (100 ng), 1 µL of Primer Perfect Probe mix and 10 µL of QuantiTect Probe PCR Master mix (Qiagen, Hilden, Germany) in each 20-µL reaction. The plates were

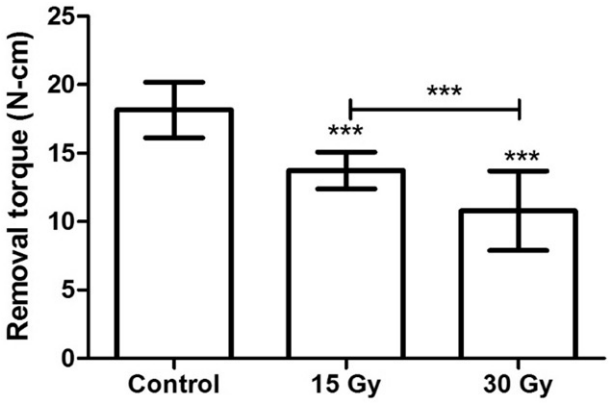

Figure 1. Mean removal torque values of dental implants placed in tibiae of rabbits (6 implants per group) without radiotherapy (control) and after irradiation with 15 or 30 Gy. ***$p < .001$.

heated for 2 min at 50 °C and 10 min at 95 °C and subsequently 40 cycles of 15 s at 94 °C and 60 s at 60 °C were run. Results are reported as relative fold-change compared to control animals. Expression data of β-actin in each sample were used as endogenous controls. Primers and probes for β-actin were designed by Primer Design (Southampton, UK). For each sample, triplicate determinations were performed in a 96-well optical plate for both targets using 9 μL of cDNA (100 ng), 1 μL of Primer Perfect Probe mix and 10 μL of QuantiTect Probe PCR Master mix (Qiagen, Hilden, Germany) in each 20-μL reaction. The plates were heated for 2 min at 50 °C and 10 min at 95 °C, after which 40 cycles of 15 s at 94 °C and 60 s at 60 °C were run. All data are reported as expression fold-change compared to expression in the control group, using the $2^{-\Delta\Delta Ct}$ method [24].

Results

Removal torque tests

The control group showed the highest mean values for removal torque (18.14 ± 2.02 N-cm). The 15 and 30 Gy irradiation groups had significantly lower torque values (13.73 ± 1.30 and 10.79 ± 2.91 N-cm, respectively) than the control group (Figure 1). The 15 Gy irradiation group had higher removal torque value than 30 Gy irradiation group (Figure 1).

Tibiae mRNA expression

A single irradiation dose of 15 Gy caused a significant decrease (0.56 ± 0.01-fold) in Bmp-2 mRNA expression in tibiae, compared to the control group (1.00 ± 0.1) (Figure 2(A)). The level of Bmp-2 in tibiae tissues that received 30 Gy irradiation was also significantly lower than in the control tibiae tissues (0.23 ± 0.03-fold and 1.00 ± 0.1, respectively). Fgf-2 mRNA expression was reduced significantly 0.64 ± 0.02-fold ($p < .001$) when rabbit tibiae were irradiated with 15 or 30 Gy radiation, compared to the control group (Figure 2(B)). In addition, the 30 Gy irradiation group had significantly lower Bmp-2 and Fgf-2 mRNA expression levels than the 15 Gy group.

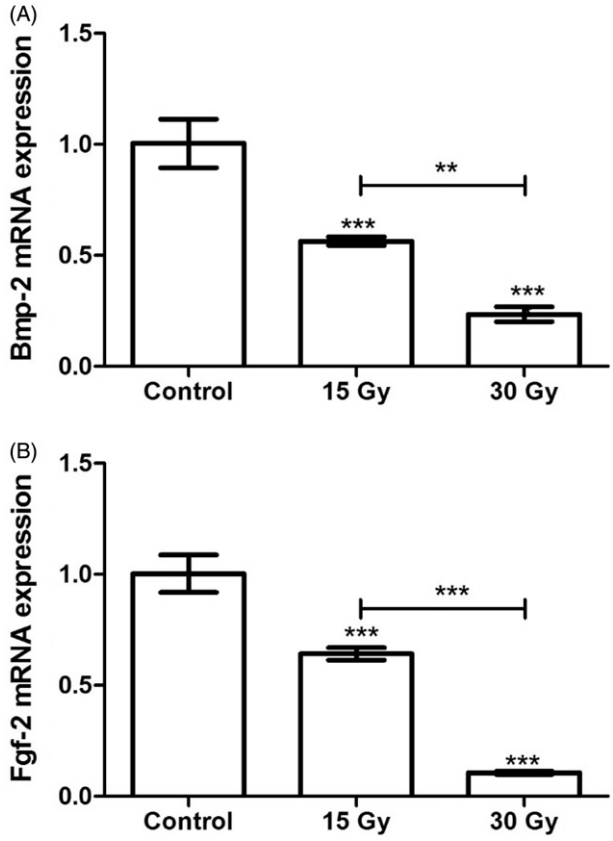

Figure 2. Relative mRNA expression levels of Bmp-2 (A) and Fgf-2 (B) in tibiae tissue of experimental rabbit groups. The expression of mRNAs was detected using quantitative qPCR analysis. β-actin was used as the reference gene. Results are reported as relative fold-change compared with control animals. Gene-specific probes were used as outlined under 'Materials and methods' section. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical comparisons were made using one-way ANOVA followed by Tukey's test. **$p < .01$; ***$p < .001$. Results are reported as means and SDs.

Discussion

In the present study, the calcified tissues formed around the implants were investigated to evaluate the dose-dependent effects of radiation on the treatment success of titanium implants. We demonstrated that 4 weeks after irradiation, removal torque values, and Bmp-2 and Fgf-2 mRNA expressions levels were lower in the irradiated groups than in the control group. In addition, the higher irradiation dose of 30 Gy resulted in a lower removal torque value and lower Bmp-2 and Fgf-2 mRNA expressions levels compared with 15 Gy radiation dose.

An interval of 4 weeks was allowed between placement of implants and radiotherapy application and another 4 weeks between irradiation and sacrifice. Because the bone turnover rate of rabbits is 3 times faster than that of human beings [25], this time was thought to be enough to evaluate the osseointegration of implants and the influence of radiotherapy.

The radiation dose is a contributing factor for the development of adverse effects in bone [26]. Tsuchiya et al. [27] used a single fraction of radiation at 10, 15 or 20 Gy doses in their experimental study and reported that bone healing is dependent on the dose. Zhang et al. [25] suggested that bone

components and maturity could be significantly impaired at radiation doses of 8.5 and 9 Gy. Muhonen et al. [28,29] showed that bone regeneration could be greatly disturbed with 60 Gy irradiation. Pohl et al. [30] observed no signs of cytotoxic effects within the time window studied using doses of 0 to 20 Gy. In our study, to compare the dose-dependent effect of radiation on implant we chose 15 and 30 Gy doses.

We found that implant removal torque values were lower in the irradiated groups than in the control group. Radiotherapy has been demonstrated to impair bone healing and regeneration [28,29,31]. Zhang et al. [25] found that after 4 weeks of consolidation, the newly formed bone in the radiation therapy groups were more immature and filled with large chondroid islands compared with the control group. In addition, micro-CT results at 1 week of consolidation showed that the bone density was significantly greater in the control group than in the irradiated group [25]. Verdonck et al. [32] indicated that at 8, 12 and 24 weeks after implant placement, in 3-month previously irradiated minipigs, a marked decrease in implant stability was observed compared to non-irradiated minipigs [32]. According to Granstrom et al. [33], irradiated sites are more susceptible to tissue necrosis and loss of implants compared to non-irradiated sites. Cao and Weischer [34] investigated the prognosis of 131 implants in 27 patients who underwent radiation therapy and showed a significantly lower implant survival rate in irradiated patients compared to non-irradiated patients after approximately 2 years of follow-up. The results of our study are in parallel with these previous studies. It can be thought that radiation impairs bone healing, and thus the stability of implants and bone-to-implant contact are affected.

In addition, we found that in the 30 Gy irradiation group, implant removal torque values were lower than in the 15 Gy irradiation group. Tsuchiya et al. [27] found that callus formation and bone regeneration were increasingly compromised in the distraction osteogenesis process with an increasing dose of radiation. Zhang et al. [25] found impaired bone healing with increasing radiation dosage. Radiation therapy in high accumulative doses can impair bone regeneration and formation during bone healing, and these adverse effects are directly dose-dependent [25]. Thus, due to such effects, in our study the stability of implants was worse at the higher dose of radiation of 30 Gy than at the 15 Gy dose.

In the present study, we additionally investigated the molecular components of bone after radiation therapy. The investigated parameters, Bmp-2 and Fgf-2 mRNA expressions, were found lower in irradiation groups than in the control group. Angiogenesis is an important aspect of bone healing and formation that is regulated by a variety of growth factors, including Bmp, Fgf, Vegf and angiogenin. BMPs are important multifunctional proteins that play essential roles in several physiologic processes such as embryogenesis, cell migration, and differentiation for osteogenesis. Among the BMP family members, Bmp-2 is closely associated with osteogenesis. Expression of Bmp-2 can be elevated during the active bone formation phase [35]. Fgf is another important growth factor involved in many physiologic processes, including angiogenesis, osteogenesis and wound healing [36]. Fgf-2 participates in osteogenesis by inducing differentiation and maturation of osteoprogenitor cells [37]. In our study, these important proteins of the bone regeneration process were found lower in the irradiation groups than in the control group. Yamamoto et al. [38] found that exogenous Bmp-2 was inhibited in the X-ray irradiated bone. Moreover, bone formation markers of ALP activity were decreased after radiation therapy [30]. Also, animal osteoradionecrosis models have shown inhibition of endogenous Bmp-2 and osteocalcin with radiation therapy [39]. Zhang et al. [31] investigated the effect of radiation therapy on bone during distraction osteogenesis and reported superior bone formation in the irradiation group than in the control group using micro-CT analysis. They also reported that that result was in line with the elevated expression of Bmp-2, -4, and -7 after radiation therapy [31]. Additionally, they found that the expression levels of Vegf and Fgf-2 were greater in the irradiation group than in the control group during active distraction and in the first week of consolidation [31]. The authors suggested that the remaining osteoblasts and endothelial cells produced more growth factors to restore normal angiogenesis and to compensate deleterious effect of radiation [31]. In addition, the higher Bmp-2 and Fgf-2 expression levels after radiation therapy in their study could be related to a stimulatory effect of the low radiation dose (8.5 and 9 Gy). However, the expression levels of Bmp-2 and Fgf-2 in our study were found decreased by higher radiation doses (15 and 30 Gy). It can be suggested that a low radiation dose could stimulate bone regeneration, but with an increasing dosage, bone regeneration could be impaired and the expression levels of Bmp-2 and Fgf-2 could be greatly decreased. Furthermore, as angiogenesis is a prerequisite for any tissue formation, lower levels of these molecules in the irradiation groups can also be related with a decrease in vascularization caused by irradiation [32].

In line with those results, our study found that radiation therapy at higher doses resulted in lower Bmp-2 and Fgf-2 mRNA expressions levels. The suppressive mechanism of radiation therapy on bone formation seems to be a result of the decreased Bmp-2 and Fgf-2 mRNA expression on cellular responses.

In conclusion, radiation can affect osseointegration of implants by reducing the quality of bone and impairing the bone-to-implant contact. At 15 and 30 Gy irradiation doses, bone regeneration is impaired and the expressions of Bmp-2 and Fgf-2 are greatly decreased. In addition, the effect of radiation is dose-dependent, as a higher dose caused lower removal torque values and lower expression of growth factors.

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Disclosure statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

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