

Original Article

Validation of a rabbit model of irradiated bone healing: preliminary report

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Abstract – Introduction: External radiotherapy can lead to severe bone alteration. The aim of this pilot study was to validate a model for assessment of postextractional bone healing in the irradiated rabbit mandible. **Material and method:** The radiation protocol consisted of 5 sessions delivering 8.5 Gy each. Surgery was performed immediately after completion of radiotherapy. Sacrifices were performed from Day 0 to Day 42. **Results:** The bone mineral density and the trabecular number were decreased after radiotherapy whereas trabecular separation increased. The main differences between irradiated and non-irradiated rabbits were observed at Day 28 and 42. **Discussion:** Radiation seems to cause a delay in bone healing. It decreases bone quality and bone mineral density. Five sessions seem to be a valuable compromise between tissues effect and feasibility of the experiment. **Conclusion:** This model seems to be valuable for evaluating postextractional bone healing in the irradiated rabbit mandible.

Introduction

The mandible is often included in the radiation fields in case of head and neck squamous cell carcinomas. External radiotherapy can lead to osteonecrosis, which still represents a threatening adverse effect that may impact quality of life [1]. Its pathogenesis is partially explained by a decrease of cell activity, a decrease of vascularization and an increase of collagen excretion, resulting in bone weakening [1]. The incidence of osteoradionecrosis has decreased to 1–5%. However, its treatment remains controversial and partially unsatisfactory and invasive (*i.e.* interruptive surgery of the mandible). Several surgical and non-surgical treatments have been proposed, like the Pentoclo[®] protocol or hyperbaric oxygen therapy [2]. Several radiation schemes have been developed in the literature, with various results [3–9]. Therefore, there is a high need of developing animal models for therapeutic testing. The aim of this pilot study was to assess the reliability and feasibility of a radiation scheme for assessing postextractional bone healing in the rabbit mandible.

Material and method

The protocol was conducted in accordance with international standards on animal welfare. It was approved by the internal ethics committee for animal experimentation of Institute of Experimental Surgery (CEEA n°10).

Twenty-two New Zealand adult female rabbits were used (age: 9–12 months; weight: 2.15–2.8 kg). Animals were randomly divided into 2 groups, one group on which only surgery was performed; and one group with radiotherapy and surgery.

The irradiation protocol consisted of 5 weekly sessions delivering 8.5 Gy each, on a linear accelerator delivering 6 MeV photons. The animals were sedated with intramuscular administration of ketamin/Xylazin/glycopyrolate 15 minutes before the session. A catheter was then placed in the vein of the ear to allow control of sedation by periodical re-injection. A scanner had been previously performed on one rabbit to calculate the dosimetry. A silicon sheet was placed to increase the thickness of the soft tissues and concentrate the maximal dose on bone. A thermoformed mattress was also used to enhance the precision of the repositioning. Before each session, a radiograph was performed to control the positioning of the animal. At the end of the session, the animals were placed under infrared light and monitored until complete

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Fig. 1. (a) Submandibular surgical approach for the creation of the bony defect. (b) Lateral view of the bony defect at D0.

recovering. The follow up was performed by daily examination, weighting and control of food and water intake. Standard limit points, defined by the ethics committee, were: fever, permanent decubitus, convulsions, severe anemia, pain symptoms resistant to analgesia procedures and anorexia superior to 24 hours.

A standardized defect (2×3 mm) created at 5 mm mesially to the mental foramen on each side of the mandible was performed immediately after completion of radiotherapy (Fig. 1a and b).

Rabbits were sacrificed from D0 (*i.e.* the day of the surgery) to D42 (*i.e.* the day of the surgery) by 4 ml intravenous pentobarbital. Samples were randomly divided in 2 groups, one for micro CT and one for histologic analysis. Analyses were performed by 2 operators.

The microCT (Inveon[®], Siemens) was performed according to standards for bone characterization in the rodent [10], with the following parameters: magnification: high, 180 projections; binning 2, pixel size: $18.76 \mu\text{M}$, axial field of view 18 mm, transaxial field of view 28.16 mm, scanning length 18 mm, voltage 80 kV, current $500 \mu\text{A}$, exposure 770 ms and acquisition 30 min. A Hounsfield and density calibration was daily performed. The region of interest (ROI) was determined and analyzed thanks to ImageJ[®] software. BV/TV (percent bone volume), TbN (trabecular number) and TbSp (trabecular separation) were reported and analyzed thanks to ImageJ[®] software. The bone mineral density (BMD) was also determined.

Samples were fixed in 10% formalin, embedded in paraffin after slow decalcification in citric acid, sliced and finally stained with hematoxylin-eosin. Criteria for histological analysis were: number and aspect of bone cells (osteoblasts, osteocytes, and osteoclasts), number and aspect of vessels, fibrosis, necrosis, and aspect of bone structure. Cell count and vascular counts were performed in 4 fields and $\times 40$ magnification.

Statistical analysis was performed thanks to SPSS (IBM, Chicago). Standard deviation was calculated and a *T* test was performed to compare the two groups in terms of BMD, BV/TV, TbSp and TbN.

Table I. Details of the groups and time interval for sacrifice.

Time for sacrifice (D : day)	Sham rabbits (number)	Irradiated rabbits (number)
D0	2	2*
D7	2	0
D14	2	0
D28	2	4
D42	2	3**

*1 rabbit died during radiotherapy, probably due to anesthesia; 1 rabbit died before arriving to the laboratory.

**1 rabbit died during surgery.

Results

Three rabbits died during the experiment: one rabbit died during radiotherapy, probably due to anesthesia; 1 rabbit died before arriving to the laboratory; and 1 rabbit died during at the end of a radiation session. Thus, it resulted in 20 samples for the control group and 18 samples for the irradiated group (Table I).

All animals gained weight (mean: 121% in the non-irradiated group and 105% in the irradiated group) during the experiment. Adverse effects observed were mild fugitive anorexia, light cough and very localized hair loss.

External radiotherapy was performed on a 6 MeV photons linear accelerator. Five weekly sessions delivering 8.5 Gy each were performed (alpha/beta ratio = 2). The total dose was 42.5 Gy which is equivalent to 98 Gy in human. Animals were sacrificed at D0, D7, D14, D21, D28 and D42 for the control group and D0, D28 and D42 for the irradiated group.

Visual examination

The defect was visible at D7, and D14 in the non-irradiated group, and visible at any time in the irradiated group. The bone appeared less mineralized and weaker.

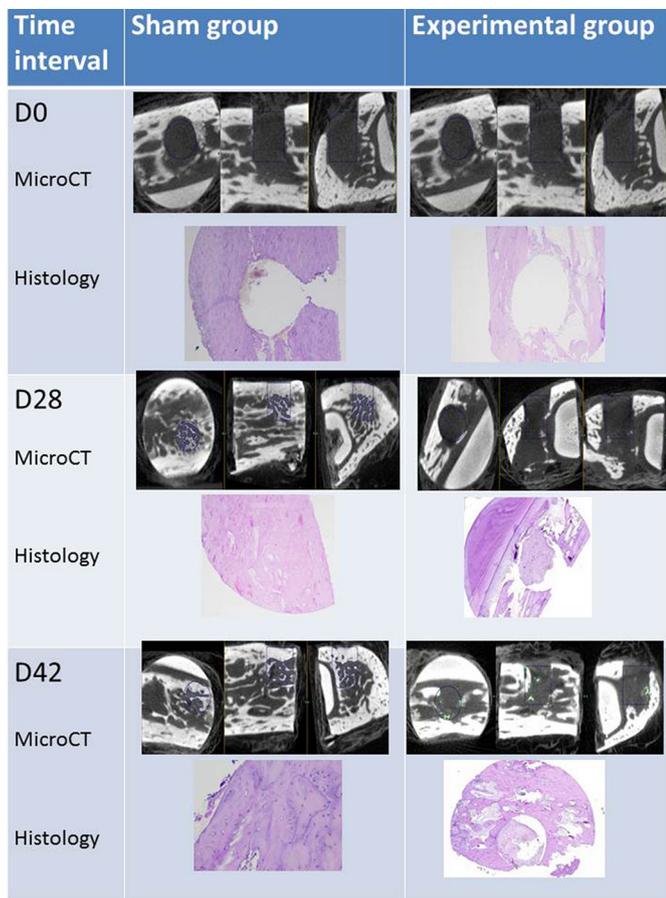


Fig. 2. Representative images of each group: comparison between microCT and histology at D0, D28 and D42. At D0, no differences can be seen between irradiated and non-irradiated samples. At D28, bone healing is almost completed in the non-irradiated samples whereas it is visible in the irradiated group. At D42, the defect is non visible in the non-irradiated group but still visible in the irradiated group with few amounts of immature bone.

MicroCT

The defect was visible at D7, and D14 in the non-irradiated group, difficult to identify at D21 and non-visible at D28 and D42. In the irradiated group, the defect was visible at any time (Fig. 2).

In the control group, BV/TV, TbN and the BMD increased over time, whereas TbSp decreased, which showed bone healing (Fig. 3). The values for BV/TV at D0, D28 and D42 were respectively 1.5, 30.6, 28.3 in the control group and 0.22, 0.91, 9.05 in the irradiated group, with statistical significance ($p=0.008$ at D28 and 0.02 at D42). For BMD, they were respectively 1050.1, 1620.2, 1868.4 in the control group and 1038.8, 1143.9, 1242.7 in the irradiated group ($p=0.03$). For TbN, they were 0.14, 0.86, 1.02 in the control group and 0.02, 0.07, 0.49 in the irradiated group ($p=0.01$ at D28 and 0.03 at D42). In the irradiated group, the increase of BV/TV and TbN values was more important between D28 and D42, whereas it was between D0 and D28 in the control group. The difference

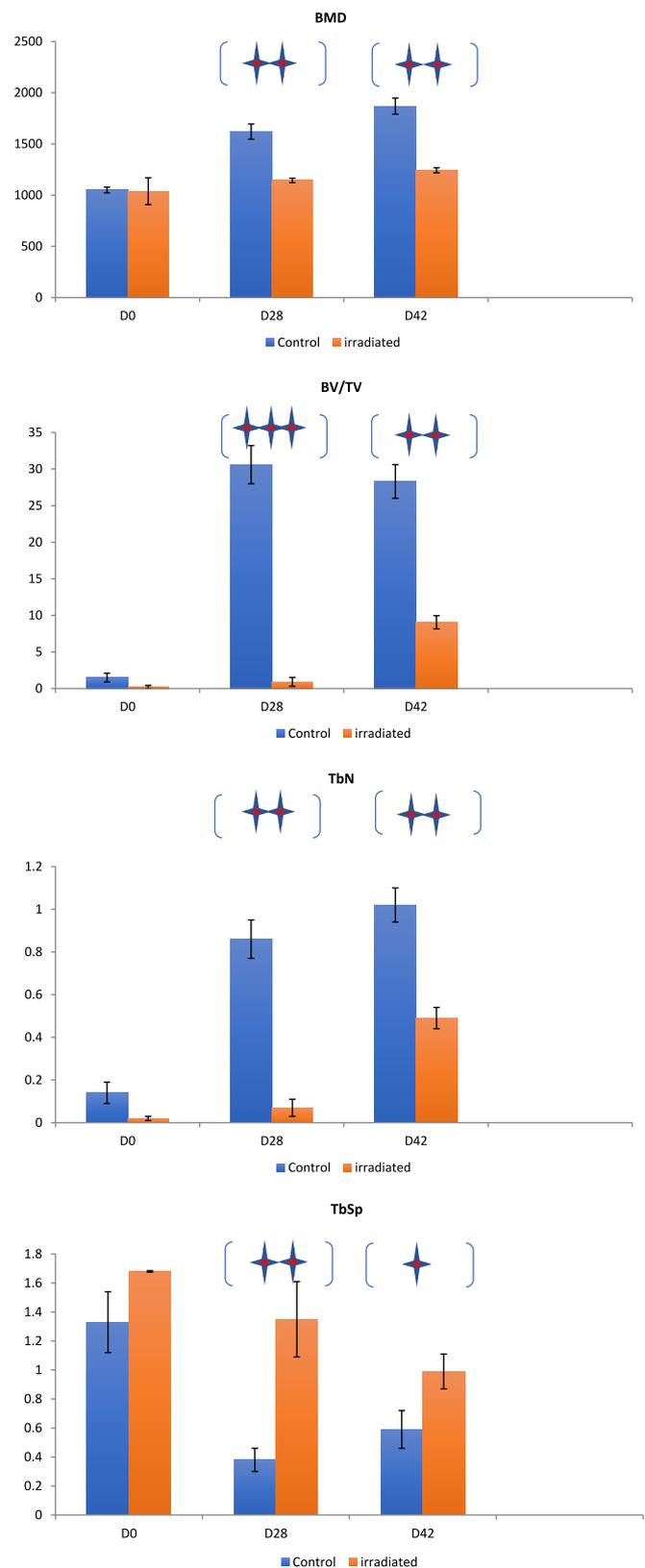


Fig. 3. Comparison between control and irradiated groups after the results of the microCT analyses at D0, D28 and D42: values for BMD, BV/TV, TbSp, TbN, Standard Deviation, and statistical significance (T test) for each value and time interval. Statistically significant results are highlighted with one red star for $p < \text{or} = 0.05$, 2 stars for $0.01 < p < 0.05$, and 3 stars for p values $p < 0.01$.

Table II. Histologic analysis at D28: cell count, vascular count and microscopic features of irradiated and non-irradiated samples. *P* value is given for each parameter.

Parameter	Non irradiated group	Irradiated group	Statistical significance
Cortical/haversian bone ratio	0/1 to 3/2	0/1 to 2/3	NS
Architectural modifications	No	No	NS
Viable osteocytes count	403.3 (354–436)	275.1 (213–318)	$p = 0.03$
Viable osteoclasts count	9.3 (5–14)	2.6 (0–6)	$p = 0.03$
Viable osteoblasts count	193.4 (143–219)	30.3 (12–47)	$p = 0.02$
Necrosis	No	Yes	S
Fibrosis	No	No	NS
Vascularization (vessel count)	19.2 (11–31)	7.4 (5–10)	$p = 0.04$

NS=the difference between irradiated and non-irradiated samples is not statistically significant. Confidence interval is determined at 95%, *i.e.* $p < 0.05$ for statistical significance.

between the 2 groups was important at D28 and D42, except for TbSp, which re-increased in the control group between D28 and D42 (Fig. 3).

Histology

For the control group, at D7 and D14, the defect was partially filled. Fibrovascular tissue and immature bone was found. At D28 and D42, the defect was completely filled by mature bone.

In the irradiated group, the defect was not completely filled, even for D42. Only fibrovascular tissue could be found, with only little area of bone in the defect. Cell counts and alterations of the vascularization were significantly different from irradiated to non-irradiated samples, with, in the irradiated group, osteocyte and vessel count divided by a factor of 2, osteoclasts count by a factor of 3.5, osteoblasts by a factor of 6.4 and vascularization by a factor of 2.6. Table II presents histologic results at D28, where differences are the most important (Table II). Adipous necrosis was noticed in irradiated samples.

Discussion

Rabbits are considered as a valuable model for initial experimentation evaluation, despite major differences. The bone turnover is highly increased and the bone marrow is fatty [3].

Animals

Rabbits did not present with usual radio-induced adverse effects, like anorexia, loss of weight, alopecia or mucositis. Zhang [5] also described a lack of any mucosal or skin adverse effects, which corroborates the need of higher doses to reproduce adverse effects similar to humans. Sacrifices at D0 were performed to ensure the initial comparability of the groups.

MicroCT

MicroCT has a great interest in the analysis of bone structure [10] as it allows the assessment of 3D bone geometry, BV/TV, trabecular thickness, number and separation. Apparent BMD can also be calculated using the Hounsfield unit scale. The parameters chosen for microCT analysis (BV/TV, TbN, TbSp and BMD) are concordant with the literature [10–12]. The problem was the identification of the ROI particularly in the control group as bone healing seemed to be almost completed at D28. Moreover, there might be a difficulty to choose the threshold parameters to separate bone trabeculae and marrow space, which may influence the images. Guyatt [13] showed that bone density is correlated to the radiation dose, the time interval between radiotherapy and examination, and the use of HBO. Comparing the control and the irradiated groups, BV/TV was divided by a factor of 30 at D28, and 3.38 at D42. BMD was divided by 1.41 at D28 and 1.5 at D42; and TbN by 12.28 at D28 and 2 at D42. The fact that BV/TV and TbN dramatically increase between D0 and D28 in the control group and between D28 and D42 in the irradiated group is concordant with the fact that it takes more time to heal in irradiated bone.

The increase of TbSp in the control group between D28 and D42 is barely explainable. Nonetheless, this increase was not statistically significant ($p=0.11$), even if resulting in very similar values between the control and the irradiated group, which seems to show that in the irradiated mandible of the rabbit, TbSp would probably be stable after 42 days of healing, whereas healing takes longer time in terms of BMD, BV/TV and TbN.

Histology

Slow decalcification seems preferable to many authors [7–9]. Histology allows a semi quantitative analysis, reporting empty lacunae, osteoblasts/osteoclasts ratio, osteocytes count, presence of woven bone, presence of osteoid volume and aspect of the vessels. After radiotherapy, there is an increase of fibrous tissue, less viable osteocytes, less

vascularization and less mature bone [3,7]. Osteocytes are decreased, and empty lacunae are reported. Osteoblasts are highly altered by external radiotherapy and a decrease of osteoblasts 3 months after radiotherapy is reported [14,15]. There are some cell nuclei changes compatible with cell death. Fibrous connective tissue is also observed. In the irradiated samples, the cortical/haversian bone ratio doesn't seem altered by previous radiotherapy, whereas there is a significant decrease of osteocytes, osteoblasts, osteoclasts and vascularization. Bone architecture seems also preserved. Endosteal osteoblasts seemed to have a relative resistance to radiation [14]. Fibroatrophy seems to be an early phenomenon [16]. There is an important inter and intra variability of histological analyses in the literature in term of techniques used, and despite semi-quantitative methods (*i.e.* cell counts), the interpretation of the results is difficult.

Outcomes

Radiation seems to delay bone healing, bone quality and bone mineral density. These observations on the mandible are also reported in long bones. On rabbit tibias, radiation-induced bone alterations were more important after a time interval of 14 to 21 days, which seems to be also relevant in rabbit mandible [4]. Thus, the study particularly focused on D28 and D42. Statistical analyses performed showed that D28 is the earliest time interval with significant effects, and probably with the most important radio-induced bone alterations.

The protocol developed by Zhang [5,9] for studying distraction osteogenesis after radiotherapy, and used in this study with 5 weekly sessions of 8.5 Gy seems to be relevant for assessment of postextractional irradiated bone healing. Five sessions seem to be a valuable compromise between tissues effect and feasibility of the experiment. This is quite different than the radiation scheme used in humans for head and neck squamous cell carcinomas, which generally consists in 20 to 35 daily sessions delivering 1.8 to 2 Gy each. Thus, bone healing is 3 times faster in rabbits [7] and bone recovery might be faster after radiation injury. In the literature, considering mandibular rabbit models, the total dose delivered was equivalent to at least 50 Gy, which is a threshold for radio-induced adverse effects [17]. To onset osteoradionecrosis, higher doses must be used (70 Gy, 90 Gy and 110 Gy eq dose [16], and should be associated with surgery : 50 Gy+surgery 2 weeks after completion of the radiotherapy in rats [18], 50 or 70 Gy and surgery 30 weeks after completion of radiotherapy in minipigs [19]. Some authors also propose a single dose radiation therapy, which is a point to be discussed [5]. Fractionated schedule seemed to be better for mimicking human radiotherapy [5]. On the contrary, single-dose irradiation did not allow the tumor cells recovery (and its differential effect between normal and tumor cells) which may mislead the clinical acute and subchronical adverse effects [20]. Data have to be cautiously interpreted [21], as a single 15 Gy dose may be mathematically equivalent to 23 sessions of 2 Gy each, but not biologically.

The dose per fraction ranges from 5.5 Gy [22,23] to 9 Gy [5,9]. Lower doses did not lead to relevant bone alterations, but it is noticeable that no surgery was performed [22,23].

In the literature, surgery is generally performed on the lateral part of the mandible, in the preangular area, as it is the only part without teeth [24], with an extra oral access. Little studies [16,20] report tooth extraction, as it is surgically risky and requires a close follow up due to possible anorexia.

Concerning the time interval between radiotherapy and surgery and/or the sacrifice, Eppley [25] considered that a time interval of six weeks in the rabbit was equivalent to a time of 18 to 24 weeks in the human. For, Zhang, one month in a rabbit's life span is equivalent to 6 months in humans. For Clark [6], rabbit's bone turnover is 3 times faster, and thus healing 3 to 4 times faster compared to humans.

Conclusion

Despite major differences between rabbit mandibular bone and human bone, and a 3 to 4 times faster bone turnover [7], the rabbit remains an interesting model [3]: it provides enough bone, observation time can be shortened. Rabbits are docile, cheap and easy to handle. They can provide enough tissue samples for studies and for surgical procedures. Therefore they seem to be an appropriate model for studying osteoradionecrosis and radiation-induced bone damages [5,9]. Performing 5 weekly sessions delivering 8.5 Gy each seems to be relevant for studying postextractional radio-induced bone alterations in the rabbit mandible. As major differences were observed at D28, this time interval seems pertinent to evaluate potential effects of biostimulating procedures. Further studies on non-invasive biostimulating methods will be performed to assess their effects on irradiated bone healing.

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