

Extracorporeal irradiation and incorporation of bone grafts

Autogeneic cortical grafts studied in rats

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The incorporation of resected, extracorporeally irradiated (1, 5, 25 and 50 kGy) and orthotopically reimplanted autogeneic cortical bone was investigated in 116 adult Wistar rats. 7 mm-long diaphyseal segments of the tibia were resected, irradiated and reimplanted using K-wire osteosynthesis. Autogeneic fresh grafts served as controls. Graft healing was evaluated by radiography and histomorphometric study at 3, 6, 9, and 12 weeks. At 3 weeks, two thirds of the 50 kGy irradiated grafts were fractured and therefore the series with this dose was interrupted because of mechanical graft insufficiency. After 3 and 6 weeks there were no statistically significant

differences among the control group and 1 or 5 kGy irradiated grafts. The healing of 25 kGy irradiated grafts was delayed from the sixth week onwards and continued until the end of the experiment at 12 weeks (50% reduction of incorporation). The incorporation of 1 and 5 kGy irradiated grafts showed a 16% (1 kGy) to 24% (5 kGy) delay at 12 weeks, compared to autogeneic fresh grafts. 1 and 5 kGy irradiated autogeneic bone grafts retain most of their biological potential. Resection, extracorporeal irradiation and reimplantation of bone tumors may therefore be a possible alternative to allografting.

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In limb-salving procedures for treatment of malignant bone tumors resection, extracorporeal irradiation and reimplantation can be applied (Uyttendaele et al. 1988). Spira et al. in 1970 were the first to use irradiation to destroy the tumor cells. Irradiation is commonly used to sterilize bone for storage in bone banks, to study the effects of different doses of irradiation on chemical components of bone matrix (Turner et al. 1956), and on the biologic property known as the bone induction principle (Buring and Urist 1967) as well as for postoperative radiotherapy in combination with bone grafts (Nathanson and Bäckström 1978, Roy-Camille et al. 1981, De Santis et al. 1990). Doses exceeding 10 kGy significantly inhibited the osteoinductive activity of demineralized bone matrix, and irradiation of mineralized bone affects its potential osteoinductive property to an even greater degree than irradiation of the demineralized matrix (Urist and Hernandez 1974).

We investigated the incorporation of extracorporeally-irradiated cortical bone in a weightbearing rat model.

Animals and methods

116 adult male Wistar rats type Chbb:Thom (SFP) weighing 300–360 g were used. Autogeneic 7 mm-long cortical grafts were harvested under general anesthesia through an anterior incision of the right tibia, using a motor-driven rotating saw subjected to a constant flood of saline solution. A teflon tube fixed by a rigid intramedullary K-wire (diameter 1.2 mm) was inserted and served as a temporary spacer. Grafts were carefully freed of periosteum and bone marrow, stored in sterile glass tubes and transported to the irradiation laboratory. The graft bones were exposed to gamma irradiation, from a ⁶⁰Co-source, to total doses of 1, 5, 25, or 50 kGy. Dry ice preservation was not necessary, because temperature in the beam exceeds room temperature by a maximum of 2 degrees. 48 hours after the first operative procedure the teflon-tube was removed and the grafts were reimplanted orthotopically. Again the graft was held in place by a K-wire providing gross axial alignment. After the operation, the animals were allowed full weight bearing. Autogeneic fresh grafts served as controls.

Table 1. Radiographic score

	Points
No healing	0
Callus formation	1
Beginning osseous union	2
Almost absent osteotomy line	3
Complete osseous union	4

Table 2. Results of radiographic scoring. Mean (SD)

	Points		3 weeks	6 weeks	9 weeks	12 weeks
No healing	0	Fresh	1.7 (0.8)	3.1 (0.7)	4.9 (0.9)	6.1 (1.2)
Callus formation	1	1 kGy	1.3 (0.9)	3.4 (0.5)	3.6 (1.0) ^a	4.5 (0.5) ^a
Beginning osseous union	2	5 kGy	1.4 (0.5)	2.7 (0.5)	3.6 (0.5) ^a	4.6 (0.9) ^a
Almost absent osteotomy line	3	25 kGy	1.3 (0.8)	2.2 (0.7) ^a	3.9 (0.9) ^a	4.1 (0.9) ^a
Complete osseous union	4					

Student's t-test: irradiation groups compared with controls
^a p < 0.05

Table 3. Cortical bone formation index. Mean (SD)

	3 weeks	6 weeks	9 weeks	12 weeks
Fresh	0.15 (0.05)	0.38 (0.11)	0.64 (0.11)	0.73 (0.11)
1 kGy	0.10 (0.05)	0.34 (0.08)	0.42 (0.07) ^b	0.61 (0.10) ^a
5 kGy	0.09 (0.05)	0.35 (0.12)	0.44 (0.16) ^a	0.55 (0.17) ^a
25 kGy	0.16 (0.08)	0.21 (0.06) ^{b,c}	0.32 (0.13) ^b	0.36 (0.15) ^{b,c}

Student's t-test: irradiation groups compared with controls

^a p < 0.05

^b p < 0.01

^c 25 kGy compared to 1 and 5 kGy, p < 0.05

Incorporation of the grafts was studied in vivo at 3, 6, 9, and 12 weeks after surgery by using radiography and the animals were anesthetized for this procedure. For reproducible images the hindlegs were fixed on a high-resolution film and radiographs were made in a mediolateral projection. Fluorochromic dye was injected subcutaneously at 3 (xylenol orange 90 mg/kg body weight), 6 (calcein 10 mg/kg body weight), 9 (tetracycline 30 mg/kg body weight) and 12 (calcein) weeks. A minimum of 7 animals in each irradiation category and in the control group were killed by an overdose of pentobarbital i.p. at 3, 6, 9, and 12 weeks 2 days after the injection of the last fluorochromic dye. The tibiae were harvested as a whole, leaving a thin cover of soft tissue intact and cut into about 2 cm-long segments, fixed in 40% ethanol, run through an alcohol series to xylol and embedded in methylmethacrylate. 5 µm-thick longitudinal sections were stained using the Goldner method and with toluidine blue. 50-80 µm-thick ground sections were prepared from each specimen for fluorescence microscopy. All sections were evaluated blindly.

In the radiographic evaluation, a score according to Lane and Sandhu (1987) was used. For each proximal and distal osteotomy, 0 up to 4 points were given, 8 points being then maximum (Table 1).

Mid-sagittal sections were taken to quantify healing, using an image analysis system. Cortical bone formation at the 4 graft-host junctions was quantified as the total area of new bone within the osteotomy

line expressed as a percentage of the total area of the osteotomy gap (cortical bone formation index). The osteocytes within the graft were also counted and the percentage of lacunae occupied by osteocytes was taken as an indicator of the revitalization of the graft. The percentage of the area of bone marrow in the medullary cavity of the graft was calculated.

The mean values and standard deviations were determined for each parameter from a minimum of 7 samples for each time period and group. Statistical analysis was performed with Student's t-test. Differences between the groups were regarded as significant for p-values < 0.05.

Results

At 3 weeks, in autogeneic fresh as well as in irradiated grafts, early callus formation was found at the proximal osteotomy sites (Table 2). This callus formation occurred predominantly at the host site and was more advanced proximally than distally. Fluorescent micrographs showed labels only in the host bone. No significant differences between the control and the irradiated groups were evident (Table 3). The marrow cavity was filled with fibrous vascular tissue. In irradiated grafts a maximum of 10% of all lacunae was occupied by osteocytes while in fresh grafts microscopically living osteocytes were found in 35% of all lacunae (Table 5). Revascularization of autogeneic

Table 4. Restitution of bone marrow. Mean (SD)

	3 weeks	6 weeks	9 weeks	12 weeks
Fresh	35 (14)	78 (16)	89 (15)	98 (1.9)
1 kGy	24 (5.0)	53 (21) ^a	71 (19)	74 (12) ^a
5 kGy	25 (5.5)	79 (12)	60 (24)	65 (22) ^a
25 kGy	28 (11)	57 (20) ^a	68 (13) ^a	73 (18) ^a

Student's t-test: irradiation groups compared with controls

^a p < 0.05

fresh grafts was first seen at the endosteal surface. Invading vessels from the periosteum were only visible in the proximal areas of callus formation (Figure 2). In irradiated grafts, no vessels were seen at 3 weeks. Minimal osteoclastic activity was evident on the host as well as on the graft site in all groups. 5 of 7 50 kGy irradiated grafts were fractured so this series was stopped at 3 weeks.

At 6 weeks, all fresh and irradiated grafts showed osseous union by woven bone at the proximal osteotomy. The direction of the trabeculae was perpendicular to the long axis in most cases. Fresh and 5 kGy irradiated grafts were revascularized from the endosteal and periosteal surfaces. Apposition of newly formed bone occurred at the endosteal surface mostly in areas of hematopoietic bone marrow and was confirmed by the presence of layers of fluorochromic dye. In 25 kGy irradiated grafts there was no revascularization. There was no evidence of massive graft resorption, but in some cases a slight increase in internal porosity was seen. The fibrous tissue in the marrow cavity was replaced by hematopoietic tissue. Remnants of fibrous marrow were found in the middle third of the grafts. The incorporation process of 25 kGy irradiated grafts was significantly delayed at 6 weeks, but 1 and 5 kGy irradiated grafts were comparable to fresh ones.

The proximal graft-host interface consisted of lamellar bone in fresh grafts whereas woven bone predominated in irradiated grafts at 9 weeks. In some cases, a nearly complete reorganization of the cortex made it difficult to identify the osteotomy. Compared to samples obtained at 6 weeks, the healing at the distal osteotomy had markedly improved. All osteotomies showed osseous union. The healing processes of 1 and 5 kGy irradiated segments were identical. The proximal osteotomies showed osseous union by woven or lamellar bone in equal parts. The ends of the grafts were revitalized, as indicated by osteocytes and ingrowth of vessels. The marrow cavity was not completely filled with red marrow (Table 4). Fibrous tissue was still found in the middle parts. Compared to 6

Table 5. Revitalization of grafts. Mean (SD)

	3 weeks	6 weeks	9 weeks	12 weeks
Fresh	35 (19)	47 (9.6)	64 (16)	77 (9.0)
1 kGy	10 (7.8) ^a	39 (14) ^a	42 (13) ^a	62 (14) ^a
5 kGy	9.4 (6.8) ^a	35 (20)	46 (9.9) ^a	54 (17) ^b
25 kGy	6.4 (8.6) ^b	20 (7.4) ^b	22 (9.0) ^{b,c}	23 (9.2) ^{b,c}

Student's t-test: irradiation groups compared with controls

^a p < 0.05^b p < 0.01^c 25 kGy compared to 1 and 5 kGy, p < 0.01

weeks, the sections obtained at 9 weeks now revealed a statistically significant delay in the healing process of all irradiated grafts.

Union at the proximal transplant-host junction was of lamellar type bone at 12 weeks (Figure 3). Compared to 9 weeks, the cortex was restituted to 73% in autogeneic fresh grafts and to 61% and 55% in 1 and 5 kGy irradiated grafts, respectively. The slight porosity of the grafts as seen at 9 weeks was absent at 12 weeks. The distal osteotomies showed osseous union in all cases, but the gaps were filled by only woven bone. Over the whole period of observation, the healing was delayed distally more than proximally (Figure 1). In fresh grafts the medullary canal was completely filled with hematopoietic bone marrow, whereas fibrous tissue was found in the middle parts of irradiated grafts. The grafts irradiated with 25 kGy showed osseous union to the host by woven bone in only half of the cases (Figure 3). The contact area was filled with fibrous tissue in the remaining sections. The overall amount of newly formed bone at the osteotomies was 36%. An osteoclastic resorption of the graft and host bone, as well as poor revascularization of the transplants, was visible (Figure 4). The revitalization of the grafts, as indicated by osteocytes, was significantly reduced in all irradiation groups (Table 5).

Discussion

The use of nonvascularized autogenous cortical bone grafts for the reconstruction of skeletal defects in tumor surgery has become common (Springfield 1987). A more advanced method is the en bloc resection of the bone tumor and reimplantation after extracorporeal irradiation (Spira et al. 1970, Uyttendaele et al. 1988). Reimplantation of such resected bone gives precise anatomic fit and helps to restore function of articulating joints. A number of experimental studies have dealt with the effects of irradiation used for sterilization of bone and bone matrix, but little is known

Figure 1. Radiographic healing of autogeneic fresh (A), 1 kGy irradiated (B) and 25 kGy irradiated grafts (C) at 3, 6, 9, and 12 weeks. No difference is evident at 3 weeks, but a slight gradual decrease in incorporation of the irradiated grafts begins at 6 weeks. All grafts were incorporated stable at 12 weeks.

about the incorporation of grafts irradiated by oncologically effective doses.

Doses of radiation used for sterilization do not destroy the bone induction properties of bone matrix (Schwarz et al. 1988, Wientroub and Reddi 1988). Furthermore radiation of 30–50 kGy even enhanced bone induction (Wientroub and Reddi 1988). By contrast, Urist and Hernandez (1974) reported denaturation and destruction of matrix morphogenetic properties by radiation sterilization. Munting et al. (1988) found a loss of about half of the inductive capacity to be comparable to our results and to the findings of the classic study by Heiple et al. (1963). In scanning electron microscopy, we found no deleterious effects of irradiation on the surface structure of cortical bone, whereas autoclaving caused a time-dependent swelling, detension and amalgamation of the fibrillary matrix (Voggenreiter et al. 1994).

Radiography revealed a significant delay in the incorporation of 25 kGy irradiated grafts at 6, 9, and 12 weeks, but no differences in the 3 irradiation doses. The radiographic results did not adequately detect the deleterious effects which were evident in micromorphological analysis.

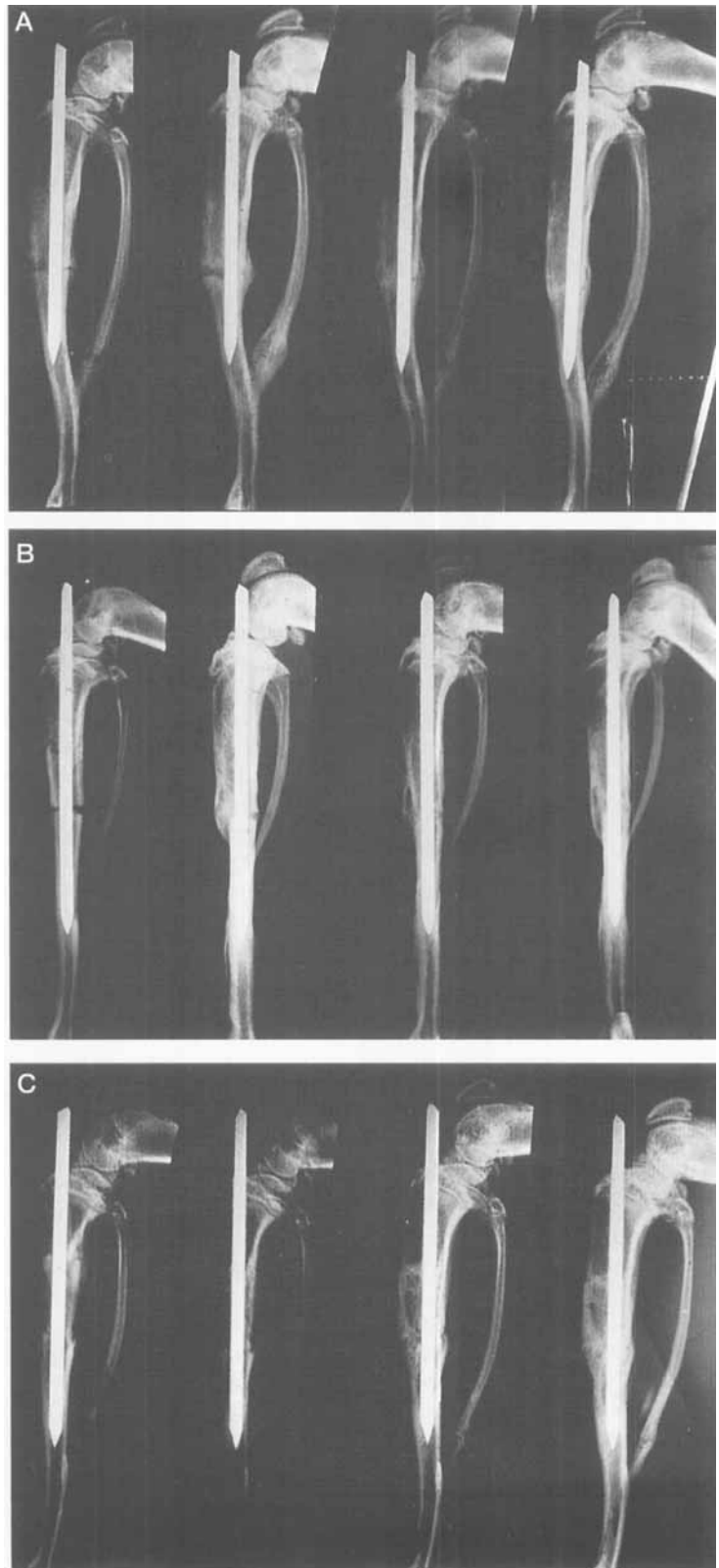




Figure 2. Early revascularization and revitalization of autogeneic fresh grafts at 3 weeks. Invasion of vessels and periosteal osteoblastic activity (Goldner's trichrome, $\times 50$).

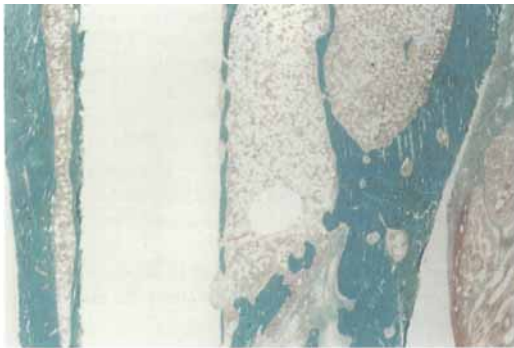


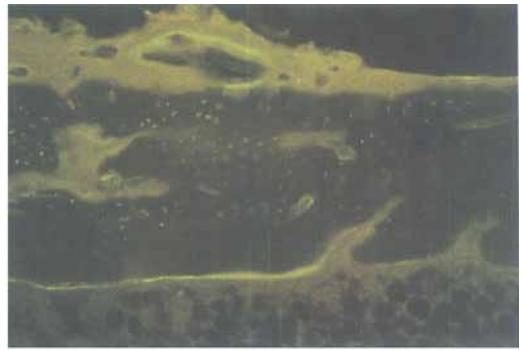
Figure 3. Excellent incorporation of 1 kGy irradiated grafts at the proximal osteotomy 12 weeks p.op. (Goldner's trichrome, $\times 50$).



In comparison the healing of 25 kGy irradiated grafts is delayed showing woven bone at the left and fibrous tissue at the right osteotomy (toluidine blue, $\times 50$).



Figure 4. Revitalization of the mid-portion of autogeneic fresh grafts at 12 weeks as indicated by the apposition of fluorochromic dyes (fluorescence microscopy, $\times 10$).



In comparison in 25 kGy irradiated grafts fluorescence is evident in only some endosteal and periosteal areas (fluorescence microscopy, $\times 10$).

A high fracture rate in 50 kGy irradiated grafts is due to a 50% reduction in the primary stability of such grafts (Voggenreiter et al. 1995). The extracorporeal irradiation of cortical bone with oncologically effective doses of 1 and 5 kGy did not substantially impair the integration of the grafts. 12 weeks postoperatively, no nonunions at the osteotomy sites were seen. Despite a slight delay in incorporation, the irradiated

grafts did not differ much from fresh grafts. Compared to incorporation of autoclaved grafts that showed nonunion in 5 of 7 animals in the study of Köhler and Kreicbergs (1987) the irradiated grafts in our study healed better. The grafts required no supplementation by demineralized bone matrix.

References

- Buring K, Urist M. Effects of ionizing irradiation on the bone induction principle in the matrix of bone implants. *Clin Orthop* 1967; 55: 225-37.
- De Santis G, Williams J F, Dvir E, McC O'Brian B, Hurley J V, Goldberg I. Effect of postoperative radiation on the incorporation of tibial bone grafts in the rabbit. *J Bone Joint Surg (Br)* 1990; 72: 309-11.
- Heiple K G, Chase S W, Herndon C H. A comparative study of the healing process following different types of bone transplantation. *J Bone Joint Surg (Am)* 1963; 45: 1593-1612.
- Köhler P, Kreicbergs A. Incorporation of autoclaved autogeneic bone supplemented with allogeneic demineralized bone matrix. *Clin Orthop* 1987; 218: 247-58.
- Lane J M, Sandhu H S. Current approaches to experimental bone grafting. *Orthop Clin North Am* 1987; 18: 213-25.
- Munting E, Wilmart J F, Wijne A, Hennebert P, Delloye C. Effect of sterilization on osteoinduction. *Acta Orthop Scand* 1988; 59: 34-8.
- Nathanson A, Bäckström A. Effects of ⁶⁰Co-γ-irradiation on teeth and jaw bone in the rabbit. *Scand J Plast Reconstr Surg* 1978; 12: 1-17.
- Roy-Camille R, Laugier A, Ruysen S, Chenal C, Bissérie M, Pene F, Saillent G. Evolution des greffes osseuses cortico-spongieuses et radiothérapie. *Rev Chir Orthop* 1981; 67: 599-608.
- Schwarz N, Redl H, Schiesser A, Schlag G, Thurnher M, Lintner F, Dinges H P. Irradiation-sterilization of rat bone matrix gelatin. *Acta Orthop Scand* 1988; 59: 165-7.
- Spira E, Brenner H T, Lubin E. Extracorporeal irradiation of malignant bone tumors. In: Chapchal G, ed. *Operative treatment of bone tumors*. Thieme Verlag, Stuttgart 1970: 136-40.
- Springfield D S. Massive autogenous bone grafts. *Orthop Clin North Am* 1987; 18: 249-56.
- Turner T C, Bassett C A L, Pate J W, Sawyer P N. Sterilization of preserved bone grafts by high voltage cathode irradiation. *J Bone Joint Surg (Am)* 1956; 38: 862-84.
- Urist M R, Hernandez A. Excitation transfer in bone. Deleterious effects of cobalt 60 radiation-sterilization of bank bone. *Arch Surg* 1974; 109: 486-93.
- Uyttendale D, DeSchraver A, Claessens H, Roels H, Berkvens P, Mondelaers W. Limb conservation in primary bone tumors by resection, extracorporeal irradiation and re-implantation. *J Bone Joint Surg (Br)* 1988; 70: 348-53.
- Voggenreiter G, Ascherl R, Blümel G, Schmit-Neuerburg K P. Effects of preservation and sterilization on cortical bone grafts—A scanning electron microscopic study. *Arch Orthop Trauma Surg* 1994; 113: 294-6.
- Voggenreiter G, Ascherl R, Früh H J, Blümel G, Schmit-Neuerburg K P. Konservierung und Sterilisation von Kortikalis—biomechanische Untersuchungen an der Ratte. *Unfallchirurg* 1995; 98: 53-8.
- Wientroub S, Reddi A H. Influence of irradiation on the osteoinductive potential of demineralized bone matrix. *Calcif Tissue Int* 1988; 42: 255-60.