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K. Shimizu · S. Masumi · H. Yano · T. Fukunaga
S. Ikebe · S. Shin

Revascularization and new bone formation in heat-treated bone grafts

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Abstract Human immunodeficiency virus (HIV) infection is one of the possible serious complications associated with bone allografts. In order to prevent infection, grafted bone is sterilized by various treatments. Heat treatment has attracted attention as a simple and practical method. We carried out a histological study of the influence of heat treatment on autogenic bone grafts. To eliminate the problem of antigenicity of grafted bone, we used autografts, not allografts. Three types of heat-treated autografts were employed: heat-treated at 60° C for 30 min, at 80° C for 10 min, and at 100° C for 5 min; as a control, fresh autografts were replaced in the rabbits' ilium. One, 2, 4 and 8 weeks after grafting, we performed microangiography and prepared two types of samples: transparent and haematoxylin-eosin (H&E) stained. Then, using an image analyzer, we quantitatively measured revascularization and new bone formation in the grafted bone. The grafts heat-treated at 60° C showed early and good revascularization and new bone formation, from 1 to 8 weeks. The grafts heat-treated at 80° C showed relatively good revascularization and new bone formation. However, the grafts heat-treated at 100° C showed unsatisfactory revascularization and bone formation, less than 40% of control 8 weeks after grafting. Therefore, heat treatment at 60–80° C does not seriously affect revascularization and new bone formation.

Introduction

Since the report of human immunodeficiency virus (HIV) infection from an HIV-seronegative donor in bone allografts (Simonds et al. 1992), safe bone grafting methods have been required. With regard to donors, setting strict selection standards and some methods such as retesting for HIV antibodies 6 months after collecting grafts (AATB 1991) or using polymerase chain reaction (PCR) (Buck and Malinin 1994), have been tried. At present, radiation in excess of 2.5 Mrad (Loty et al. 1990; Hernigou et al. 1993) is used for sterilization. However, these tests and treatment are associated with time and economic problems, and thus, only large bone banks can carry them out. Recently, heat treatment at 60°–80° C has attracted attention as a simple and practical method, but its influence on bone grafts has not yet been investigated. The purpose of this study is to evaluate the effect of heat treatment by measuring revascularization and new bone formation quantitatively. To exclude any influence of immunogenicity of grafted bone, we used autografts, not allografts in this experiment.

K. Shimizu (✉) · S. Masumi · H. Yano · T. Fukunaga · S. Ikebe
S. Shin
Department of Orthopedic Surgery, Oita Medical University,
Hasama-machi, Oita 879-5503, Japan
Tel.: +81-97-549-4411
Fax: +81-97-549-6047

Materials and methods

Eighty mature Japanese white rabbits weighing 3.0–3.5 kg were used. They were injected intravenously with sodium pentobarbital 25 mg/kg body weight as an anaesthetic. Using an oscillating saw, full-thickness grafts of 5 × 10 mm were removed from the iliac bone. These grafts were then heat-treated by placing them in beakers containing physiological salt solution and maintained in homeothermal tanks at 60° C for 30 min, at 80° C for 10 min and at 100° C for 5 min. As a control, fresh autografted bone samples were kept in physiological salt solution at 20° C for 10 min. These grafts were then transplanted into the defects in the ilium and were fixed with 1.0 mm Kirschner wires. At 1, 2, 4 and 8 weeks after grafting, the rabbits were killed with a lethal dose of pentobarbital after microangiography using India ink.

Microangiography was carried out under anaesthesia by inserting a cannula into the aorta and injecting physiological salt solution to which 5000 units of heparin had been added. Phlebotomizing the lower vena cava, we injected 150 ml of India ink under 130 mmHg injection pressure in a mercury tonometer when the ejected blood became clear. The grafted bone was removed with the surrounding tissue and fixed in 10% neutral buffered formalin, and the ilium was then sliced for samples. These slices were decalcified, and two types of specimens were made, transparent and haematoxylin-eosin (H&E) stained.

Quantitative analysis of revascularization

A microsaw was used to cut a transverse section from the central area of grafted bone. After decalcification, we made it transparent using the Spalteholz technique. Then we observed the revascularizing blood vessels that had penetrated the grafted bone from the recipient bone. We used an image analysis device (NEXUS 600, Kashiwagi, Tokyo) and worked out the revascularization rate by calculating the ratio of the area of blood vessels in the grafted bone to that in the recipient bone.

$$\text{Revascularization rate} = \frac{\text{Area of blood vessels in grafted bone}}{\text{Area of blood vessels in recipient bone}} \times 100$$

We investigated 5 cases each at the 1st, 2nd, 4th and 8th weeks after grafting. The results were statistically analysed using a *t*-test. Specifics of the image analysis method at our facility have already been reported (Yano et al. 1993).

Histological findings

Transverse sections were resected out of the central areas of grafted bone as H&E-stained samples. After histological observations were made of the inside of the grafted bone, we compared the areas of grafted bone and new bone using image analysis. We then worked out the new bone formation rate by calculating the ratio of the area of new bone to the area of bone trabeculae in the grafted bone.

$$\text{New bone formation rate} = \frac{\text{Area of new bone in grafted bone}}{\text{Area of bone traveculae in grafted bone}} \times 100$$

We investigated 5 cases each at the 1st, 2nd, 4th and 8th weeks. The results were statistically analysed using a *t*-test. For the image analysis, we used the same system as for the analysis of revascularization.

Results

Quantitative analysis of revascularization

One week after transplantation, the control group showed rapid revascularization, and the 60° C processed group evidenced only a slight delay (Fig. 1). The 80° C processed group showed slight revascularization (7.7%), but the 100° C processed group showed no revascularization. Two weeks after transplantation, revascularization had reached 72.5% in the control group and 65.6% in the 60° C group. It was slightly delayed in the 80° C group (51.6%) and markedly delayed in the 100° C group (16.4%). By the 4th week, the progress of revascularization was slightly delayed in every group. At the 8th week, revascularization of the control group was close to that of the recipient area (90.2%); it was 86.7% in the 60° C group, 70.8% in the 80° C group, and 35.6% in the 100° C group. The delay in revascularization in the 100° C group was noticeable (Figs. 2, 3).

Histological findings and quantitative analysis of new bone formation

One week after transplantation. In the control and 60° C groups, the penetration of granulation tissue with blood vessels into the graft from the recipient bone was seen at an early stage (Fig. 4). The new bone formation rate was, respectively, 3.8% and 2.3%. In the 80° C group, it was only 0.9%. In the 100° C group, neither new bone nor penetration by granulation tissue was seen. The cortical and cancellous bone cells of all three types of heat-treated bone disappeared, but their bone structures were retained. Similarly, all bone marrow tissues degenerated and necrotized, notably so in the 100° C group, where in some areas, a coagulation necrosis was found.

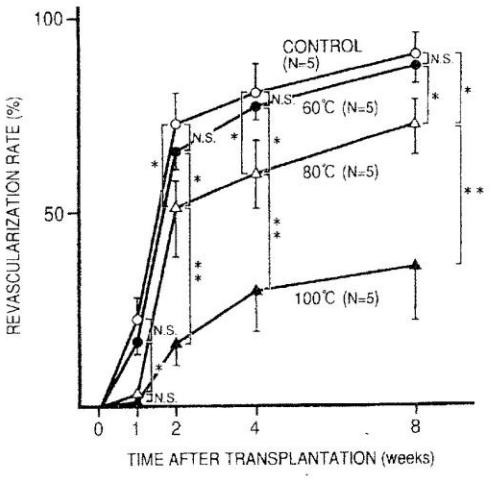

Fig. 1 Mean and standard deviation of the revascularization rate of each type of graft: *NS* Not significant, * $P < 0.05$, ** $P < 0.01$

After 2 weeks. In the control, 60° C and 80° C groups, new bone had formed around the grafted bone trabeculae, and granulation tissue had penetrated into the graft. The new bone formation rate was, respectively, 12.5%, 10.9% and 8.8%. In the 100° C group, though granulation tissue was found penetrating into the edge of the graft, new bone formation was only 1.7%.

After 4 weeks. In the control, 60° C and 80° C groups, appositional new bone formation was increasingly noted. The results of the quantitative analysis were, respectively, 25.4%, 23.8% and 19.4%. In the 100° C group, most of the trabeculae and bone marrow were necrotic, and penetration of connective tissue was scarcely found; the new bone formation rate was 7.3%, which was found at the edge.

After 8 weeks. In the control group, new bone was found even in the central area of the graft bone, and the result of the quantitative analysis was 30.2%. The bone marrow tissue found between the bone trabeculae was almost normal. The new bone formation of the 60° C and 80° C groups had also increased, and the rates were, respectively, 28.4% and 22.3% (Fig. 5). In the 100° C group the new bone formation rate was only 12.5%. Coagulated, necrotized bone marrow tissue was still present, and it was preventing penetration by the granulation tissue and appositional bone formation (Fig. 6).

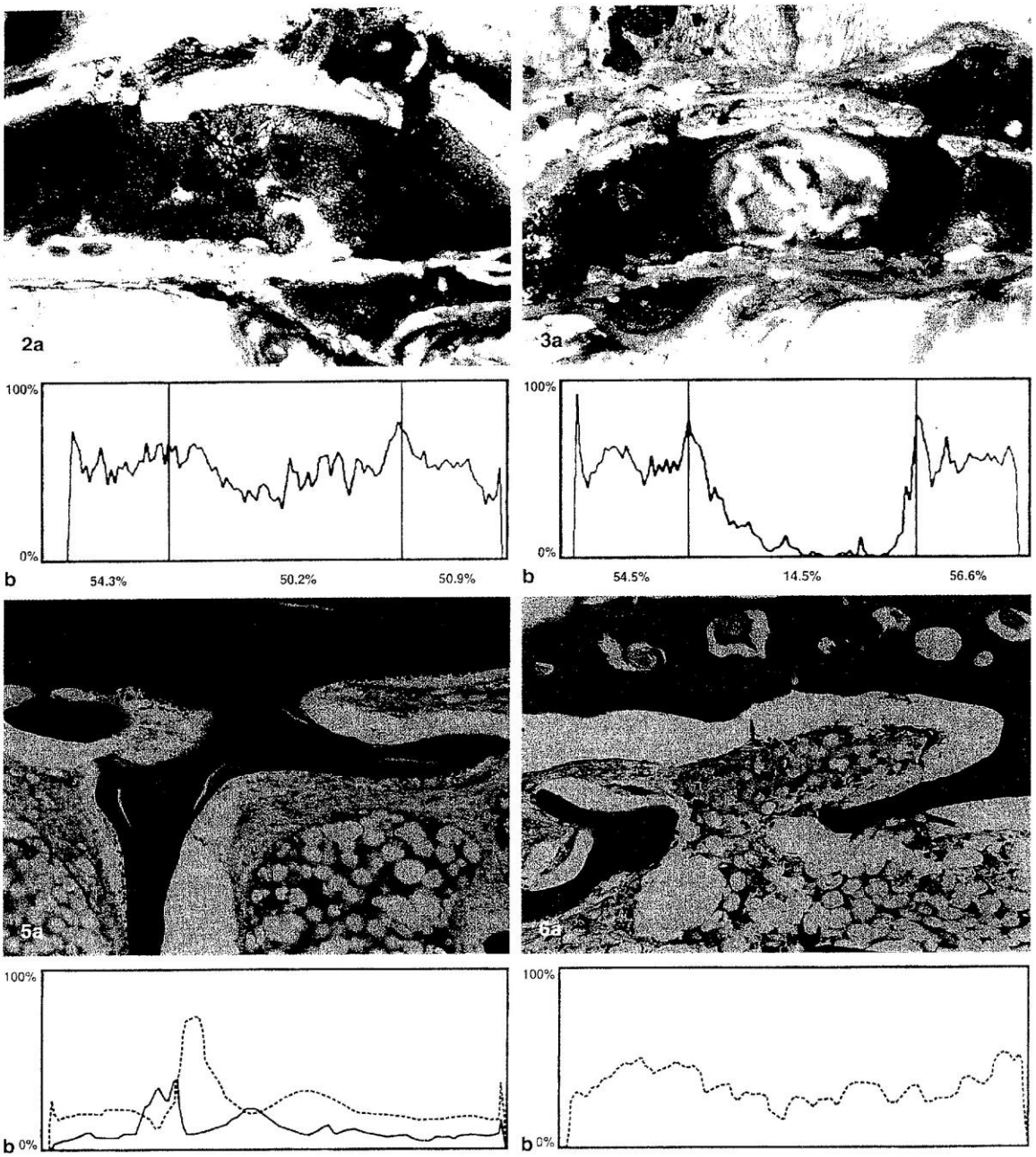

Fig. 2a, b At 8 weeks after transplantation of a graft heat-treated at 60 °C for 30 min, good revascularization is seen from the bilateral recipient site. **a** Transparent specimen (× 16). **b** Graph illustrating the ratio of the blood vessels to recipient and grafted area

Fig. 3a, b At 8 weeks after transplantation of a graft heat-treated at 100 °C for 10 min, revascularization is disturbed in the graft. **a** Transparent specimen (× 16). **b** Graph illustrating the ratio of the blood vessels

Fig. 5a, b At 8 weeks after transplantation of a graft heat-treated at 80 °C for 10 min, new bone is well formed even in the centre of the graft (haematoxylin-eosin, × 200). **b** Graph illustrating the ratio of the new bone area and grafted bone trabeculae to the total area: ——— new bone, – – – – – grafted bone trabeculae

Fig. 6a, b At 8 weeks after transplantation of a graft heat-treated at 100 °C for 10 min, new bone has not formed in the centre of the graft (H&E, × 200). **b** Graph illustrating the ratio of the new bone and grafted bone trabeculae

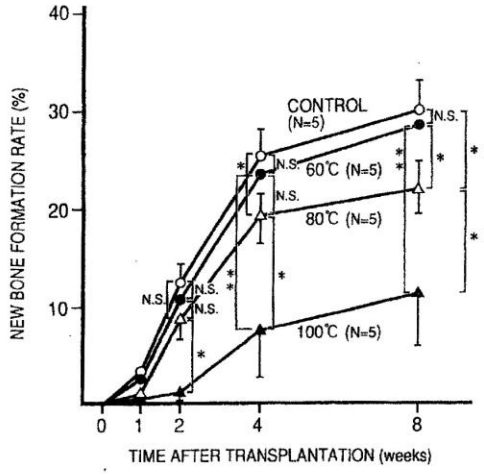

Fig. 4 New bone formation rate in each type of graft (mean and SD): *NS* Not significant, * $P < 0.05$, ** $P < 0.01$

Discussion

It was assumed that HIV infection from donors to recipients would not occur following bone allografting as the graft had been preserved at –80° C but not treated in any particular way. This is because there are few blood components in bone. However, the first case of HIV infection from a bone allograft was reported in the USA (Center for Disease Control 1988), and it was then confirmed that HIV existed in the bone and bone marrow of infected people (Folks et al. 1988; Buck et al. 1989). After that, some measures were taken, for example, strict selection of donors, and HIV antibody tests were carried out when the graft bone was extracted. Nevertheless, an HIV infection occurred through bone allografts from a donor whose HIV antibody test was negative (Simonds et al. 1992). After that, a second HIV antibody test was carried out 6 months after the extraction of graft bone from living donors (AATB 1991), and the polymerase chain reaction(PCR) method (Buck and Malinin 1994) came to be used for cadaver donors. In the former case, donors are often unwilling to take the second test (Müller and Patsalis 1994). This method is also inconvenient because grafted bone cannot be used soon after extraction, and so it is not practical for small bone banks. Moreover, an HIV-infected person who had a very long window period has been reported (Imagawa et al. 1989), so the method is not 100% reliable. As for the PCR method (Eisenstein 1990), it is not suited for living donors because it is expensive (Tomford 1993). Since screening tests have such problems, various kinds of sterilization and disinfection methods for grafted bone have been developed. Ethylene oxide gas has been used, but there are problems with its tissue osmosis (Prolo et al. 1980). Radiation requires as much as 2.5 Mrad to inactivate viruses, which demands a large and expensive facility.

Because HIV has a low resistance to heat (McDougal et al. 1985; Spire et al. 1985; Quinnan et al. 1986; Lelie et al. 1987), heat-treated bone allografts at 60°–80° C have already been clinically tried (Kuhne et al. 1994; Knaepler et al. 1994). Since the heating process requires only a small facility and is a simple method, it is practicable for small bone banks in hospitals. The basic research of heat-treated bone grafts done with animal experimental models is as follows: heat-treated and decalcified bone was transplanted to the inside of muscle layers (Inokuchi et al. 1991; Nakanishi et al. 1992), which showed the favourable bone formation ability of heat-treated grafted bone. Two experiments have addressed the histological examination of bone grafts. First, a portion of cancellous bone from the femur of a rabbit was heat-treated at 65° C for 24 h, freeze-preserved, and then used for allografts (Kühne et al. 1992). Second, the tibia of a rat was heated at 80° C for 10 min and used for autografts (Knaepler et al. 1992). Both of these experiments showed a favourable incorporation of the heat-treated bone grafts. However, these experiments had no objective quantitative evaluations.

For this study, we conducted a detailed histological examination of heat-treated bone grafts. In particular, we made quantitative evaluations of revascularization and new bone formation to show the process of incorporation of the graft. To eliminate any influence of antigenicity of the grafted bone, we used autografts, not allografts. The results showed that revascularization and bone formation tended to decrease as the temperature of heat treatment rose. This tendency was low below 80° C, but it increased strikingly at 100° C. It seems that this effect is caused by degeneration of the cytokinins concerned with blood vessel and bone formation, such as transforming growth factor beta (TGF-β) and bone morphogenic proteins (BMP). In addition, penetration of blood vessels into the intertrabecular area was physically prevented by coagulation necrosis of the bone marrow. We think that bone conduction was delayed by this coagulation necrosis. In conclusion, our quantitative evaluation showed that bone grafts heat-treated at 60°–80° C were histologically excellent with respect to revascularization and new bone formation.

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