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Morphological changes of autoclaved autogenic bone implantation and autoclaved autogenic bone supplemented with allogenic demineralized bone matrix in rat parietal bone

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Summary. The healing process of resected, autoclaved (121 °C, 20 minutes) and re-implanted bone in the rat parietal bone was compared with that of autoclaved bone that was supplemented with allogenic bone matrix (AAA-bone), using a scanning electron microscope and a light microscope.

In the implant without AAA-bone, bone union and replacement of the autoclaved bone was seen at 2 weeks after implantation. There was no evidence of any inflammatory reaction around the autoclaved bone. The implant was gradually replaced by the new bone. In the implant with AAA-bone, the new bone formation around the implanted bone was more abundant than that of the implant without AAA-bone. An inflammatory reaction was also observed after 1 week. The replacement of the implant with AAA-bone was inferior to the non-supplemented group. The reason for the poor replacement was the disturbance of the blood supply in the implant by abundant new bone formation.

In these results, the autoclaved bone re-implantation was an excellent bone substitute with osteoconductive ability and biocompatibility. The implantation with AAA-bone was good for the new bone formation, but the position and the technique of supplement with AAA-bone have to be more deeply investigated.

Key words: Bone implantation, Autoclaved bone, Scanning electron microscope, Rat

Introduction

The treatment of malignant oral tumors often involves an extensive resection of the mandibular bone. As a result, it leads to considerable problems in function and shape of the oral and maxillofacial region. In recent years, various bone implant material have been for the reconstruction of mandibular bone defects as well as fresh autogenous bone transplantation, but problems such as conferring shapes and the quantity of implant materials have still not been solved.

Reconstructed tumorous bone reimplanted after autoclaving (121 °C, 20 min) has been reported to offer a simple method for both tumor devitalization and reconstruction with suitable size and shape (Smith and Simon, 1975; Johnston et al., 1983; Kreicbergs and Köhler, 1987). The major problem of reimplantation of autoclaved bone was its low osteogenic capability, because the autoclaving of the bone caused cell death and extensive denaturation of proteins, including the bone morphogenic proteins (BMP) (Köhler and Kreicbergs, 1987). This problem has been solved by supplementation with demineralized allogenic bone matrix (Köhler and Kreicbergs, 1987; Kreicbergs and Köhler, 1989). A series of these experiments has been carried out by using long bones with cartilageous ossification.

The purpose of the present study was to investigate the usefulness of the reimplantation of autoclaved bone in the membrane bones. We used both a scanning electron microscope and a conventional light microscope.

Materials and methods

Animals

Forty-four male SD rats (aged 8 weeks) were used in this study. Sixteen rats were used for the reimplantation of autoclaved bone alone, and another sixteen rats were used for the reimplantation of autoclaved bone supplemented with a chemosterilized antigen-extracted autodigested alloimplant (AAA-bone; Urist et al., 1975). As a control, fresh autogenous bone transplantation was carried out in twelve rats.

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Preparation of AAA-bone

For the AAA-bone, long bones from two male Lewis strain rats (aged 8 weeks) were used. They were anesthetized by intraperitoneal injection of sodium pentobarbital (25 mg/Kg) and then sacrificed by transcardial perfusion with normal saline solution. After the long bones were resected, the AAA-bone was prepared according to Urist et al. (1975). The AAA-bone, was stored in a vacuum bottle in a refrigerator (4 °C).

Implant procedure

The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (25 mg/Kg), and then their parietal bones were exposed by ablation of the periosteum through a dermal incision. A small area of the parietal bone (measuring approximately 2 mm in diameter) was cut off using a dental bur mounted on a low-speed dental drill with utmost care to avoid perforation of the dura mater. During bone resection, the surgical field was continuously irrigated with sterile saline solution to reduce thermal damage. After rinsing in a normal sterile saline solution, the resected bones were autoclaved at 121 °C for 20 minutes (Ritter Speedclave. Futaba Co. LTD, Tokyo, Japan). Then the autoclaved bones were reimplanted in 16 rats without fixation. In another 16 rats, the implant was supplemented with AAA-bone as particles in the osteotomy gaps and around the implant area. For the control, the same sized parietal bone was resected under anesthesia and rinsed in a normal sterile saline solution for 10 minutes to remove the blood. Then the resected bone was re-transplanted in the same place of each calvaria as fresh autogenous bone transplantation. All implanted material was finally closed by skin sutures.

After the operation the animals were fed conventional commercial food pellets (CE-2, Clea Japan, INC, Tokyo, Japan) and kept under optimum conditions (room temperature 22 °C; humidity 55%; lighting 300-500 lux; bad small less than 20 ppm).

Tissue preparations

At 1, 2, 4 and 8 weeks after implantation, animals of each group were anesthetized by intraperitoneal injection of sodium pentobarbital, and sacrificed by transcardial perfusion followed by fixation in 1.25% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 20 minutes at room temperature. The implanted area with peripheral host bone and soft tissue was removed.

For scanning electron microscopy, organic substances of the specimens were dissolved in 5% sodium hypochloride for 20 minutes at room temperature. The specimens were rinsed in 0.1 mol/L phosphate buffer (pH 7.4) three times. They were postfixed in a 1% osmium tetroxide solution in 0.1 mol/L phosphate buffer (pH 7.4) for 90 minutes and dehydrated by a graded ethanol series. After immersion in isoamyl acetate, specimens were critical-point dried with liquid carbon dioxide, mounted on stubs, coated by gold in a vacuum device, and examined with a scanning electron microscope (S-400, HITACHI Co., LTD, Tokyo, Japan).

For light microscopy, the specimens were rinsed in 0.05 mol/L phosphate buffer (pH 7.4) three times after fixation. They were decalcified in 5% ethylendiamine tetraacetic acid at room temperature for 7 days, and embedded in a water-soluble plastic media (JB-4, Polysciences INC, Warrington, USA), and 3 µm-thick serial sections were made. The sections were stained with hematoxylin and eosin and observed with a conventional light microscope.

Results

Control (fresh autogenous bone transplantation)

1 week after transplantation

In scanning electron microscopic observation, newly-formed bone, which grew from the host bone surface and covered the transplanted bone, joined to a part of the transplanted bone on the dura mater side (Fig. 1a). The new bone showed morphological features of both spongy-like bone with a lot of vascular spaces (Fig. 1b), and mostly matured bone forming the surface (described by Boyde, 1972). On the skin side, a small quantity of new bone was observed on the host side and there was no calcified union between the host and the

Fig. 1. Scanning electron micrographs (SEMs) at 1 week autogenous bone transplantation. **a.** The transplanted bone (T) is united to a part of the host bone by new bone (N). Arrows: bone union. **b.** High magnification of the new bone in Fig. 1a. The new bone shows a spongy-like appearance with a lot of vascular spaces (arrows). a, x 27; b, x 300

Fig. 2. SEM at 2 weeks after the autogenous bone transplantation. The bone union shows spongy-like bone (arrows) and has a fibrous appearance (arrow heads) on the skin side. H: host bone; T: transplanted bone. x 27

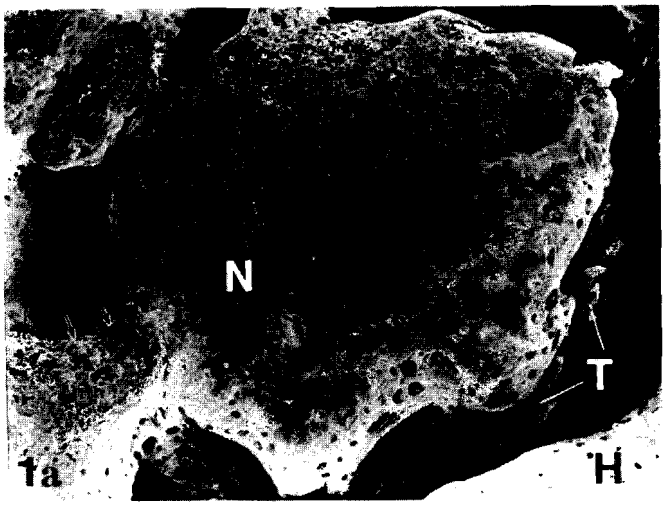
Fig. 3. Light micrograph (LM) at 2 weeks after autogenous bone transplantation. The upper part of the figures shows the skin side and the lower part is the dura mater side. The new bone (N) is formed beneath the periosteum in the dura mater side. H: host bone.;T: transplanted bone. x 62

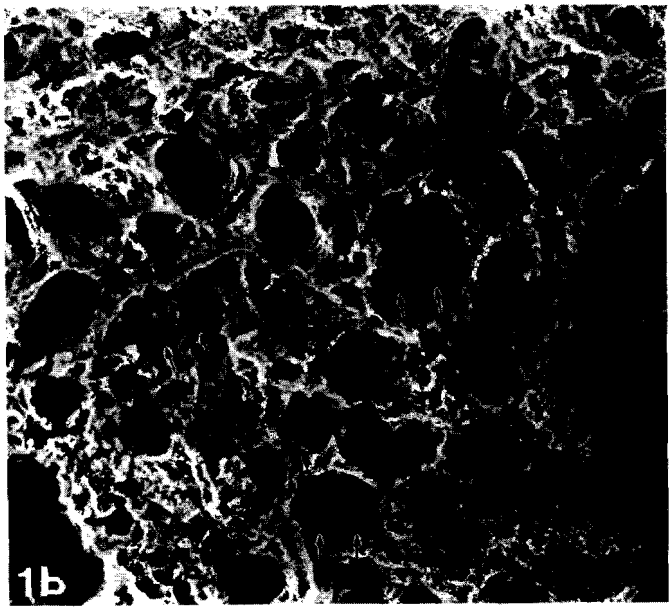
Fig. 4. SEM at 4 weeks after the autogenous bone transplantation. The bone unions (arrows) by matured new bone (N) are seen in the contact area. x 30

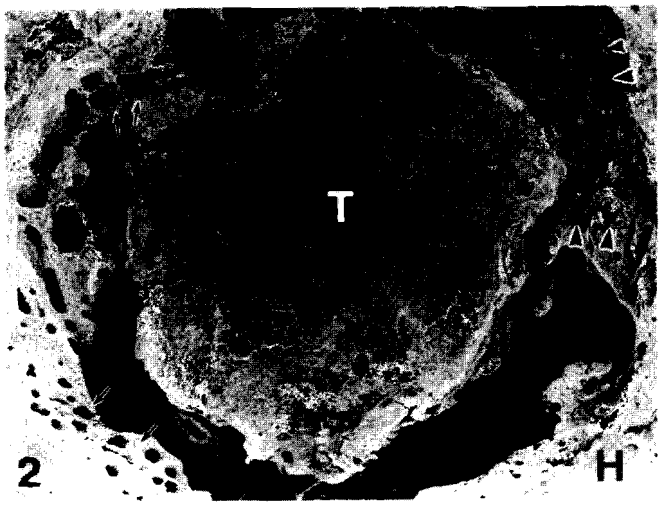
Fig. 5. LM at 4 weeks after the autogenous bone transplantation. The upper part of the figure shows the skin side and the lower is the dura mater side. New bone (N) covers the host (H) and the transplanted bone (T) and a perfect bone union (arrows) can be seen. x 62

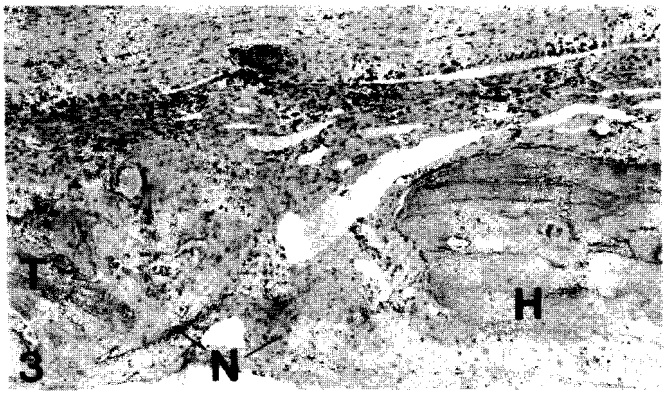
transplanted bone.

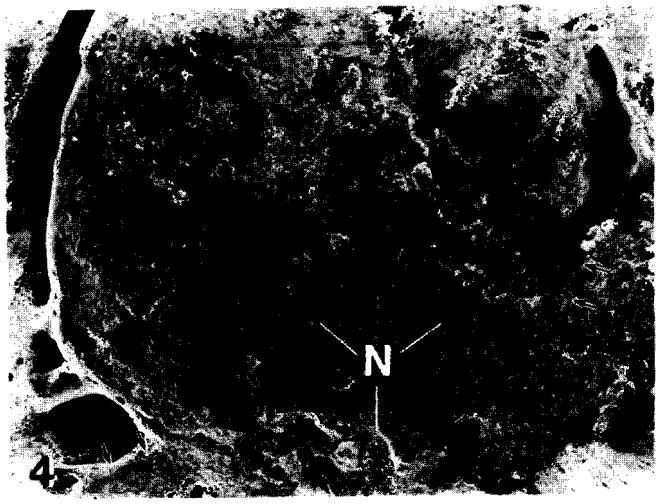
Under a light microscope, trabecular-like new bone, which was enclosed by a layer of active osteoblasts, was observed beneath the periosteum of the dura mater side. The new bone formation on the skin side was poor. There were no inflammatory reactions of the

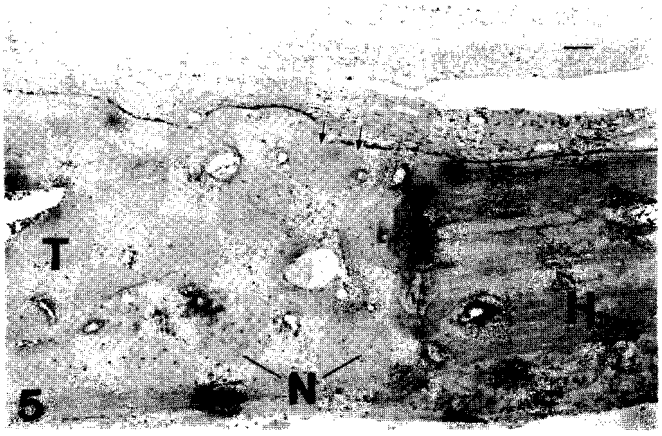












circumference of the transplanted bone.

2 weeks after transplantation

Under a scanning electron microscope, the bone union was observed in the dura mater side in a manner similar to 1 week after transplantation. On the skin side, bone union, which showed spongy-like bone and fibrous appearance, was seen in the contact area of the host and transplanted bones, but there was no new bone formation on either bone surfaces (Fig. 2).

In a light microscopic observation, new bone was seen to contact with the host and the transplanted bone beneath the periosteum, mainly on the dura mater side (Fig. 3). On the skin side, new bone was also formed on the host bone. At the contact area of both bones, new bone surrounded the cut surface. Bone-resorbed lacunae (Howship's lacunae) were observed near the cut surfaces.

4 weeks after transplantation

The scanning electron microscope showed that the incorporated area made by the new bone multiplied in the contact area of both bones in the dura mater side, but not at all contact areas (Fig. 4). The new bone showed the morphological features of mature bone and it was impossible to distinguish new bone from pre-existing transplanted bone or host bone. The bone resorption was observed on the new bone surfaces. On the skin side, incorporation areas were observed extensively.

Light microscopically, the host and the transplanted bone were surrounded by new bone in the dura mater side and a perfect bone union was seen (Fig. 5). The osteoblasts surrounding the new bone were flatter than the cells at 1 and 2 weeks after transplantation.

8 weeks after transplantation

Under the scanning electron microscope, the transplanted area in the dura mater side was covered by matured new bone. In the observation from the skin side, there were specimens which looked line non-union bone showing a deep gap between the host and the transplanted bone (Fig. 6a). In these specimens, the incorporation was completed on the dura mater side. The morphological feature of the new bone was that of further matured bone with both forming and resorbing surfaces (described by Boyde, 1972) (Fig. 6b).

At light microscopic observation, the host and transplanted bone were completely covered by matured new bone and bone union was also accomplished.

Autoclaved bone implantation

1 week after implantation

Scanning electron microscopically, there was no calcified union between the host and the implanted autoclaved bone; after inorganic treatment, the implanted bone was detached from the implant bed. The new bone was formed on the host bone on the dura mater side just like the control, and protruded towards the implant bed (Fig. 7). The new bone showed a spongy-like appearance with a lot of vascular spaces. New bone formation was not seen on the skin side.

2 weeks after implantation

Under a scanning electron microscope, on the dura mater side, the implanted bone and the host bone were joined by the spongy-like new bone, which was formed on the host bone and covered the implanted bone or filled in the contact area (Fig. 8). The new bone showed various outer forms like a spongy-like bone and flat matured bone. There was little new bone formation on the skin side.

Light microscopically, the autoclaved bone was stained a purple color, in spite of hematoxylin and eosin staining. The new bone was formed beneath the

Fig. 6. SEM at 8 weeks after the autogenous bone transplantation. **a.** The transplanted bone is perfectly covered by matured new bone (N). The bone union is mostly complete but a deep gap (arrows) can be seen in a part of the contact area. **b.** High magnification of the new bone in Fig. 6a. The new bone surface is divided into «forming» and «resorbing» surfaces, which means the remodelling of bone. F: forming surface; R: resorbing surface. a, x 27; b, x 120

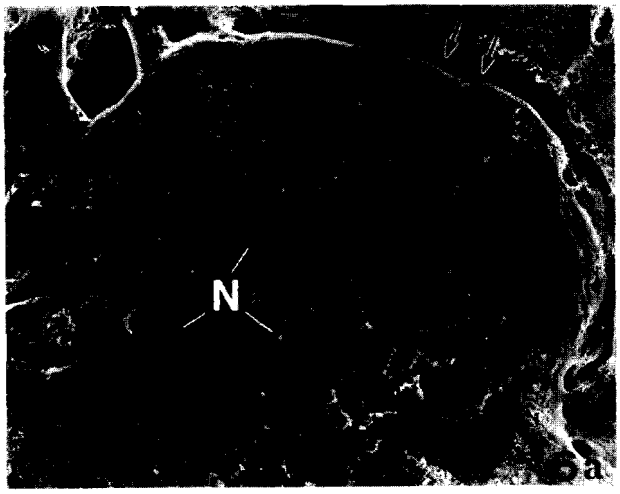
Fig. 7. SEM at 1 week after the autoclaved bone implantation (non-supplemented with AAA-bone). The observation is from the skin side. No implanted bone can be seen in the implant bed. New bone (N) protrudes towards the implant bed from host bone on the dura mater side. H: host bone. x 23

Fig. 8. SEM at 2 weeks after autoclaved bone implantation (non-supplemented with AAA-bone). Observation from the skin side. The bone union by spongy-like new bone (arrows) is seen in the contact area between the host (H) are implanted bone (I). x 23

Fig. 9. LM at 2 weeks after autoclaved bone implantation (non-supplemented with AAA-bone). The upper part of the figure shows the skin side and the lower is the dura mater side. New bone (N) is seen beneath the periosteum on the dura mater side and intrudes into the contact area. H: host bone; I: implanted bone. x 62

Fig. 10. SEM at 4 weeks after autoclaved bone implantation (non-supplemented with AAA-bone). Observation from the dura mater side. New bone (N) covers the host and the implanted bone, and bone union (arrows) is seen in part. x 23

Fig. 11. LM at 4 weeks after autoclaved bone implantation (non-supplemented with AAA-bone). The upper part of the figure shows skin side and the lower is the dura mater side. The new bone (N) breaks into the implanted bone (I) with plenty of vascular canals. v: vascular canals. x 62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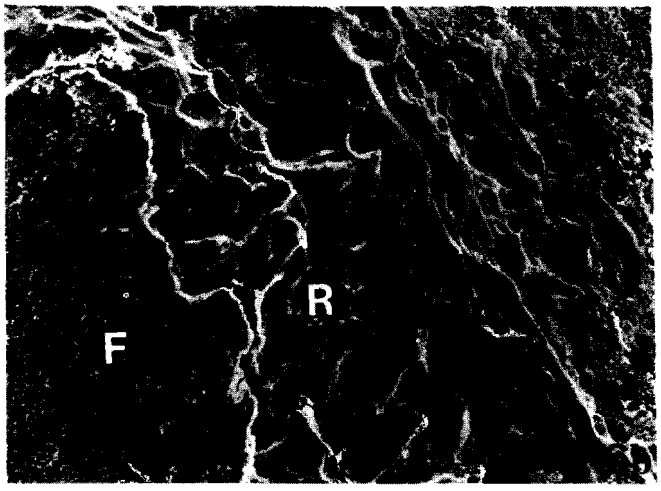
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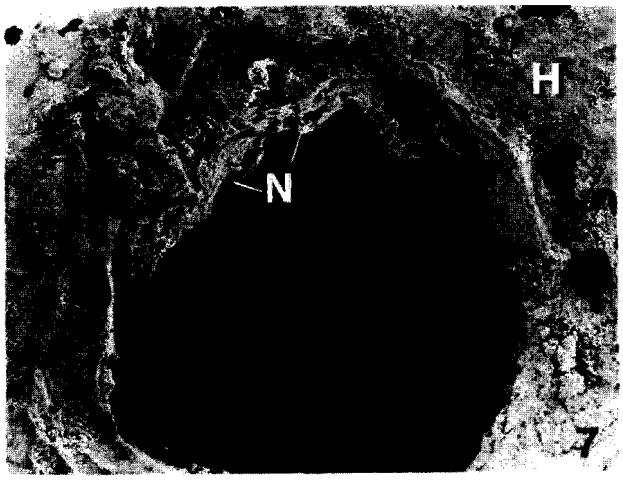
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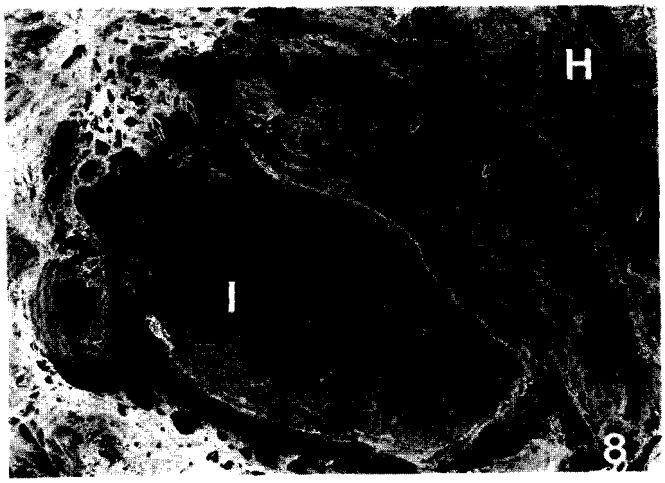
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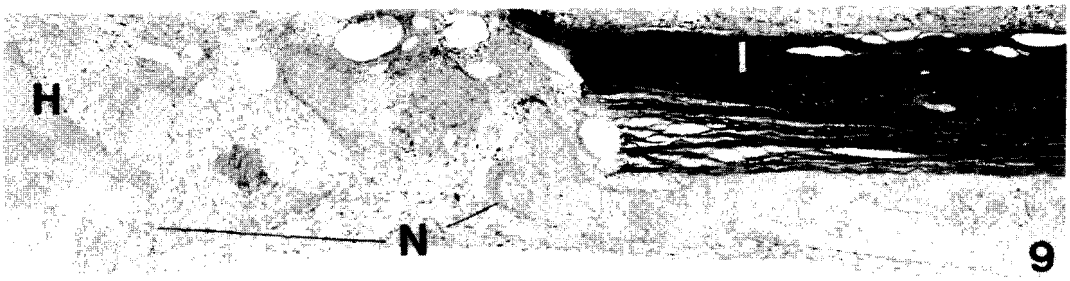
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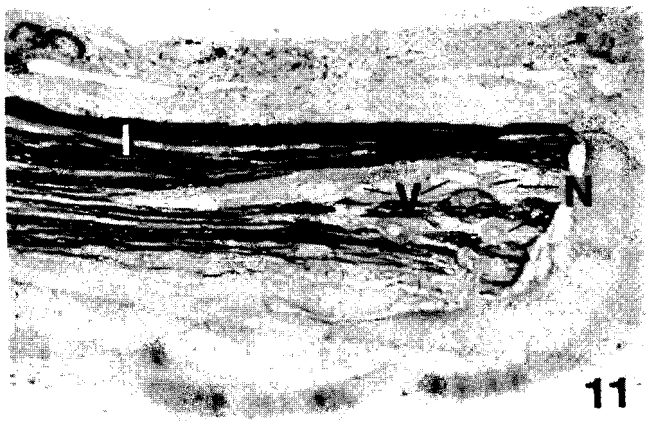
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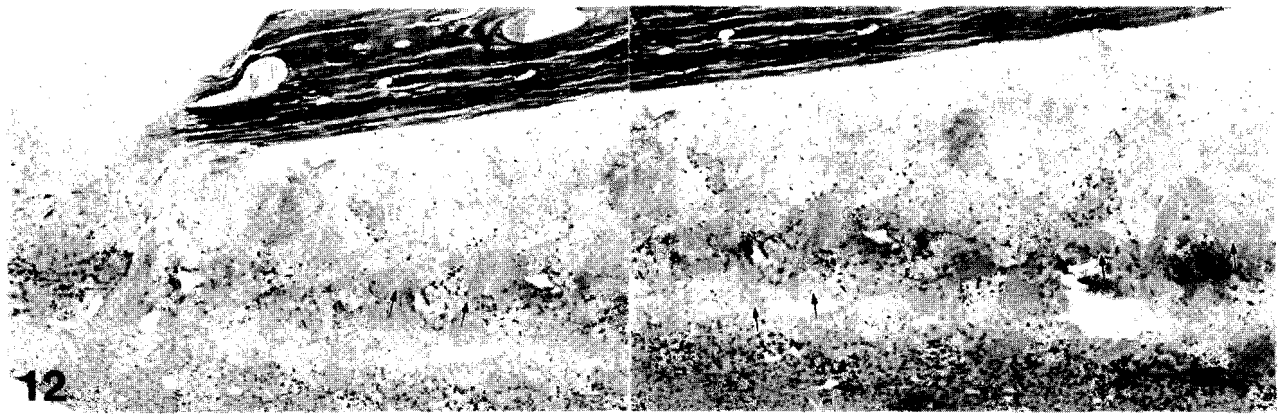
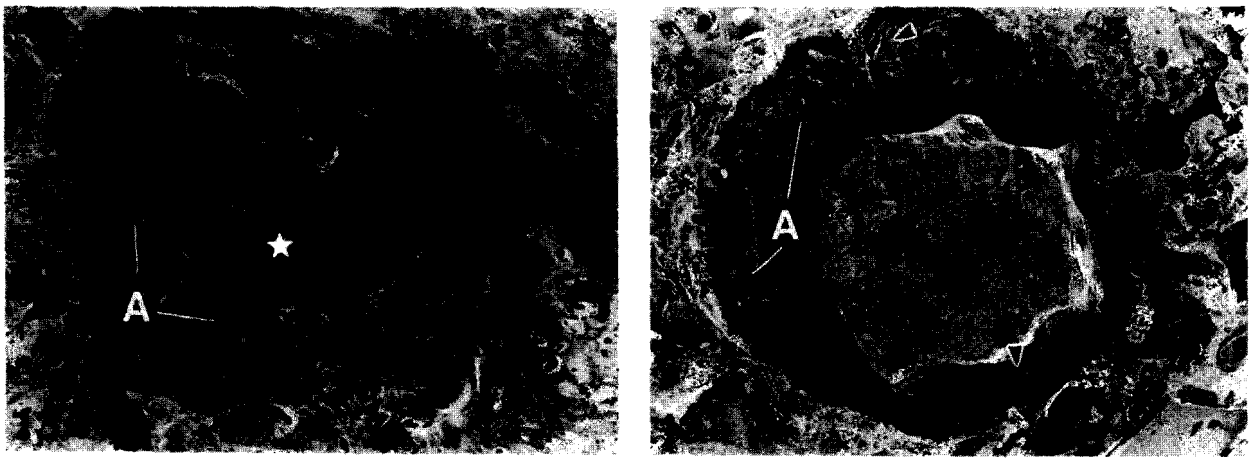
periosteum of the host bone on the dura mater side (Fig. 9). The new bone intruded into the contact area, covering the cut surface of both bones. There were no inflammatory changes around the implant.

4 weeks after implantation

The scanning electron microscope showed that the matured new bone partly connected the host bone with





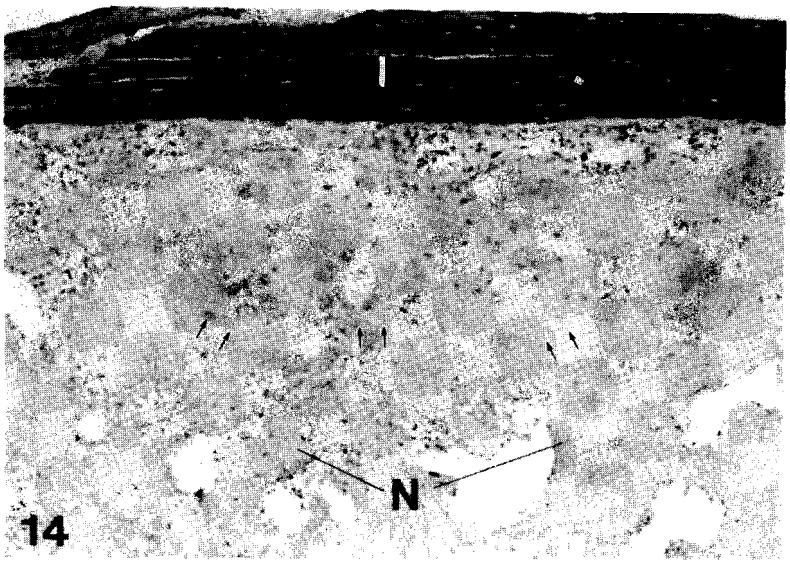


Fig. 12. LM at 1 week after autoclaved bone implantation (supplemented with AAA-bone). The upper part of the figure shows skin side and the lower is the dura mater side. The supplemented AAA-bones (arrows) are scattered in the dura mater side. There are many inflammatory cells and multinucleated cells, but no new bone formation. I: implanted bone. x 62

Fig. 13. SEMs at 2 weeks after the autoclaved bone implantation (supplemented with AAA-bone). **a.** SEM of the dura mater side. The implanted bone is covered by half matured new bone, spongy-like bone and fibrous structure. The bone union is almost completed. Star marks: implant area. A: supplemented AAA-bone matrix. **b.** SEM of the skin side. Extensive bone resorption (R) can be seen on the host bone surface. The contact area is filled by AAA-bone matrix (A) and calcified fibrous structures (arrow heads). S: spongy-like bone. a and b, .x 20

Fig. 14. LM at 2 weeks after autoclaved bone implantation (supplemented with AAA-bone). The upper part of the figure shows the skin side and the lower is the dura mater side. The new bone (N) with cartilage (arrows) is formed on the dura mater side. I: implanted bone. x 62

the implanted bone and covered both bones on the dura mater side (Fig. 10). On the skin side, the cut surface of the host bone was covered by matured new bone, which was of a low grade of maturity with many vascular spaces, and the bone union was observed in part.

Light microscopically, the bone union was accomplished in the form of new bone to fill the contact area. The new bone broke into the implanted bone with plenty of vascular canals (Fig. 11). Multinucleated large cells were also seen around the vascular canals.

8 weeks after implantation

The results of the scanning electron microscope were similar to those at the same periods of the control. The bone union was perfected on the dura mater side, if not accomplished in parts of the skin side.

Light microscopically, both host and implanted bones were covered by the new bone and the bone union was completed. The implanted bone was partly replaced by new bone.

Autoclaved bone implantation with AAA-bone

1 week after implantation

Scanning electron microscopically, there was no calcified union between the host and the implanted bone; after inorganic treatment, the implanted bone was detached from the implant bed. There was no bone formation on the skin side. New bone was formed only

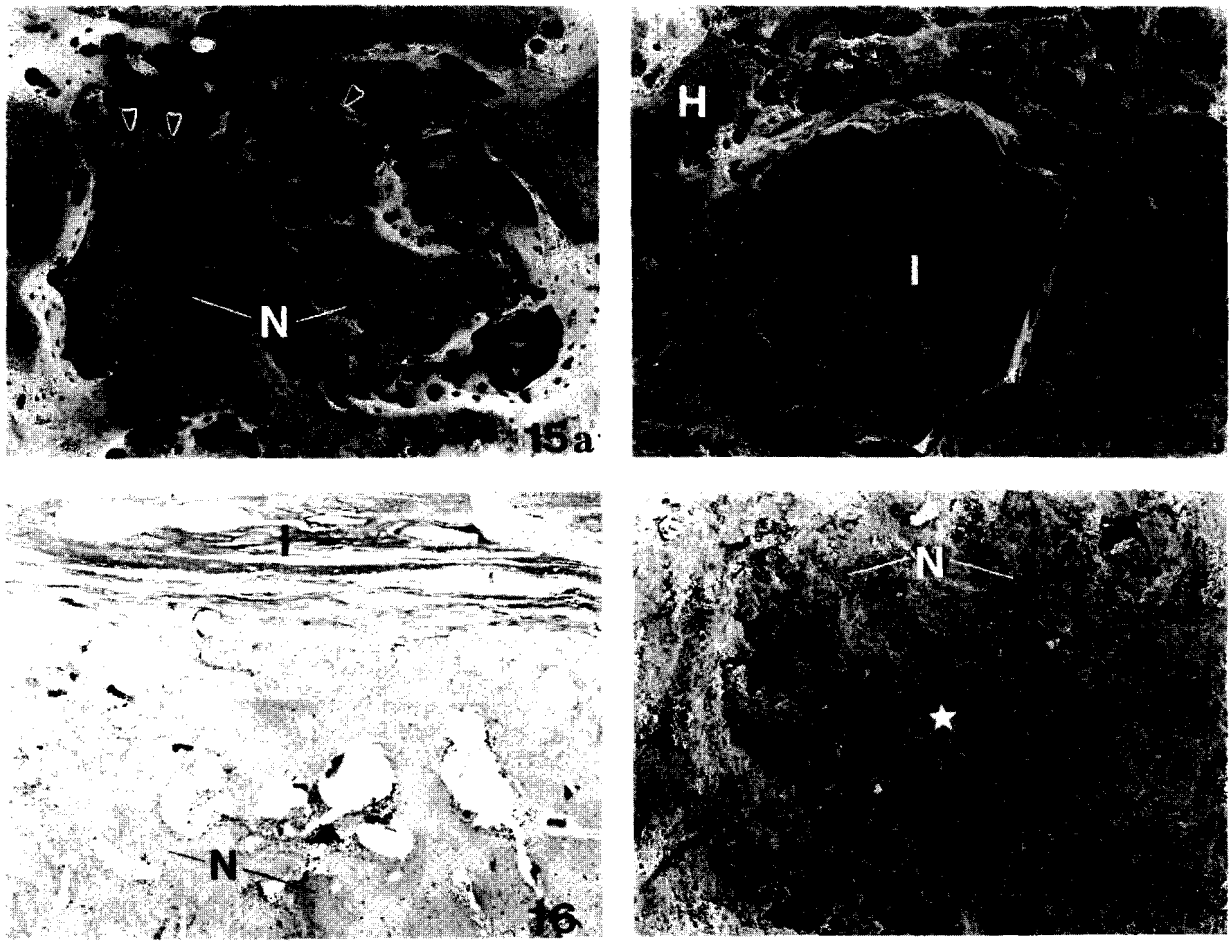


Fig. 15. SEMs at 4 weeks after autoclaved bone implantation (supplemented with AAA-bone). **a.** SEM of the dura mater side. AAA-bone matrix (arrow heads) is still observed, but the new bone (N) shows a matured appearance and covers the implanted bone. **b.** SEM of the skin side. The bone union can be seen in almost all contact areas. H: host bone. I: implanted bone. a and b, x 20

Fig. 16. LM at 4 weeks after autoclaved bone implantation (supplemented with AAA-bone). The upper part of the figure shows the skin side and the lower is the dura mater side. New bone (N) surrounds the implanted bone (I) but does not enter into the implanted bone. x 62

Fig. 17. SEM at 8 weeks after autoclaved bone implantation (supplemented with AAA-bone). Observation of the dura mater side. It is impossible to distinguish pre-existing host and implanted bone from the new bone (N). star mark: implant area. x 22

on the host bone on the dura mater side.

In light microscopic observations, new bone enclosed by osteoblasts was formed on the host bone on the dura mater side. The aggregation of undifferentiated mesenchymal cells was seen in the circumference of the new bone. The supplemented AAA-bones were seen scattered on the dura mater side of the implanted bone with many inflammatory cells and multinucleated cells (Fig. 12). There was no new bone formation around the AAA-bone.

2 weeks after implantation

The scanning electron microscope showed that the new bone on the host bone came close to the matured bone with little vascular space. On the implanted bone, spongy-like bone which connected to the matured new bone on the host bone was observed. Calcified fibrous structures were also observed among the supplemented AAA-bone (Fig. 13a). There was no new bone formation in the greater part of the host bone surface on the skin side, except for a small area accompanied with spongy-like bone (Figs. 13b). Extensive bone resorption existed on the cut surface of the host bone. The contact area of both bones was filled by the supplemented AAA-bone matrix, and calcified fibrous structures covered the matrix.

Light microscopically, the new bone with active osteoblasts was formed beneath the periosteum of the host bone on the dura mater side. Around the implanted bone, plentiful new bone with cartilage was formed from outside the AAA-bone matrix to the dura mater (Fig. 14). The new bone around the AAA-bone connected to the new bone on the host side. There were no inflammatory cells.

4 weeks after implantation

Scanning electron microscopically, bone union was seen in almost all of the contact areas of both bones, in contrast to the case of the control group and the non-supplemented autoclaved bone implant (Fig. 15a). The morphological appearance of the bone came near to that of matured bone, although there were many vascular spaces (Fig. 15b). The supplemented AAA-bone matrix was still observed in partial areas, but there were no calcified fibrous structures.

In light microscopic observations, new bone formation on the dura mater side was extremely vigorous (Fig. 16). The implanted bone was covered by new bone and the bone union was complete for most parts. Blood vessels and fibrous tissues came into the implanted bone, but there was no new bone formation.

8 weeks after implantation

Under the scanning electron microscope, it was impossible to distinguish the host bone from the implanted bone in both skin and dura mater sides (Fig. 17).

Light microscopic observations were similar to the findings at 4 weeks after implantation. There were no remarkable changes in the inside of the implanted bone.

Discussion

The incorporation of bone transplantation and/or implantation is defined by the osseous fusion between the host and the implanted bone (Kreicbergs and Köhler, 1989). The osseous fusion is the most important phenomenon for the permanence of bone reconstruction.

In the comparative study of the autoclaved bone implantation and the autoclaved autogenous bone supplemented with demineralized allogenic bone matrix (DABM) implantation on the ulna of adult rabbit, the resected, autoclaved, and reimplanted diaphyseal segments supplemented with DABM incorporated better than non-supplemented implants of autoclaved autogenous bone (Köhler and Kreicbergs, 1987). In this study using rat parietal bone, which is a membrane bone, non-supplemented implants of autoclaved autogenous bone showed favorable incorporation as well as implant with chemosterilized antigen-extracted autodigested alloimplant (AAA-bone). With regard to the style of the incorporation, in the scanning electron microscopic observations, both non-supplemented and supplemented with AAA-bone implants of autoclaved autogenous bone joined and are covered by new bone, in contrast to the control group, in which the bone union is seen in the contact area of the host and the implanted bone. This style is similar to the results of the recent studies on freeze-dried bone implantation and sintered bone implantation employing the same animals and methods (Matsuda et al., 1992, 1995). It is noteworthy that the new bone infiltrates into the implanted bone of the non-supplemented implant in the early stage of the implantation as compared with the supplemented with AAA-bone implant.

An abundant blood supply in the implanted bone is the most important phenomenon for good osseous fusion and replacement, that is to say «creeping substitution» (Ray, 1972). The autoclaved bone is «an inert spacer» because the autoclaving of bone results in the degeneration of various bone proteins and the autoclaved bone has no osteoinductive ability (Köhler and Kreicbergs, 1987; Kreicbergs and Köhler, 1987). It is thought that the structure of the bone changes after autoclaving and the autoclaved bone has a rough structure. The autoclaved bone, therefore, gives the host tissue favorable circumstances for the creeping substitution.

AAA-bone and DABM is an excellent bone inductive substance (Urist et al., 1975; Janovec and Dvorak, 1988). In this study, new bone formation in the implant supplemented with AAA-bone was rich, but the invasion of blood vessels into the implanted bone and the replacement by new bone were poor. The reason for the disturbance of the blood supply and the poor

replacement is thought to be the inadequate position of the supplement with the AAA-bone rather than the host reactions, as compared with the result of the non-supplemented implant. As a result of the abundant new bone formation induced by the AAA-bone, the invasion of blood vessels into the implant would be disturbed and the replacement would be delayed. The supplemental position and the supplemental techniques with the AAA-bone have to be investigated further.

The physical properties of autoclaved bone were studied using the diaphyseal bones of adult rabbits (Köhler et al., 1986). In the results, the autoclaved bone shows a moderate decrease in strength, stiffness and weight, but remains mechanically adequate for skeletal substitution. It is also clear from this study that the autoclaved autogenous bone implant is easily accepted by the host tissue. The size and shape of this material are the same as those of the bone defect, and autoclaving can bring the tumor cells in tumorous bone to a complete physiological and dynamic studies for the oral functions such as mastication are need to be applied to the maxillofacial bone, especially to the jaw bone. When these problems are solved, the autoclaved autogenous bone must be a useful material for facial bone implants.

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