Influence of Particle Size of Autogenous Bone Grafts on the Early Stages of Bone Regeneration: A Histologic and Stereologic Study in Rabbit Calvarium

Lars Pallesen, DDS[1]/Søren Schou, DDS, PhD[2]/Merete Aaboe, DDS, PhD[3]/
Erik Hjørting-Hansen, DDS, Dr Odont[4]/Anders Nattestad, DDS, PhD[2]/Flemming Melsen, MD, Dr Med[5]

Purpose: *The aim of this study was to investigate the influence of particle size of autogenous bone grafts on the early stages of bone regeneration.* ***Materials and Methods:*** *Bicortical skull bone defects were prepared in 15 rabbits (4 in each rabbit). Two defects were filled at random with either small (0.5 to 2 mm³) or large (10 mm³) autogenous bone particles. In addition, 1 defect was left unfilled (control defect). All defects were covered bicortically by expanded polytetrafluoroethylene membranes. The animals were divided randomly into 3 groups and sacrificed after 1, 2, and 4 weeks, respectively. Histologic and stereologic evaluations were performed after the sections were blinded.* ***Results:*** *No significant differences in total vessel surface area could be identified among the 3 groups. The total volume of newly formed bone in defects with small particles was larger and more mature compared to defects with large particles after 2 and 4 weeks. Furthermore, the resorption of small particles was more pronounced after 4 weeks, documenting a higher level of bone substitution compared to large particles.* ***Discussion:*** *The early stages of bone regeneration were influenced by the particle site of autogenous bone grafts.* ***Conclusion:*** *The present study indicated that particles of 0.5 to 2 mm³ in size should be preferred to particles of 10 mm³ in size for bone grafting.* (Int J Oral Maxillofac Implants 2002;17: 498–506)

Key words: *autogenous bone grafts, bone regeneration, calvarial defects, histology, particle size, particulated bone grafts, rabbits, revascularization, stereology*

Bone grafting is frequently used in oral and maxillofacial surgery for reconstruction of the alveolar process before implant placement.[1–3] In addition, treatment of congenital and traumatic defects as well as jaw reconstruction after extensive tumor or cyst removal often involves bone grafts.[4–7] The ideal bone graft should be osteoinductive to stimulate osteogenesis and osteoconductive to provide a scaffold for establishing optimal conditions for ingrowth of blood vessels and cells with osteogenic potential. These requirements are presently most adequately fulfilled by autogenous bone grafts alone or in combination with xenografts.[5,8]

Autogenous bone grafts can be used in blocks or in a particulate form.[7] Particulated bone grafts are often preferred to blocks because it is expected that there will be more pronounced revascularization around the graft particles and a larger release of growth and differentiation factors from the graft in the early stages. In addition, the total surface area of particles is much larger than that of a block graft. Consequently, osteoclastic activity is facilitated, resulting in more resorption. However, the present knowledge about the most appropriate bone particle size for achieving

[1]Research Associate, Department of Oral and Maxillofacial Surgery, School of Dentistry, Faculty of Health Sciences, University of Copenhagen, Denmark.
[2]Associate Professor, Department of Oral and Maxillofacial Surgery, School of Dentistry, Faculty of Health Sciences, University of Copenhagen and University Hospital (Rigshospitalet), Copenhagen, Denmark.
[3]Senior Resident, Department of Oral and Maxillofacial Surgery, Copenhagen County University Hospital, Glostrup, Denmark.
[4]Professor and Chairman, Department of Oral and Maxillofacial Surgery, School of Dentistry, Faculty of Health Sciences, University of Copenhagen and University Hospital (Rigshospitalet), Denmark.
[5]Professor, Department of Pathology, Aarhus County Hospital, Aarhus, Denmark.

Reprint requests: Dr Søren Schou, University of Copenhagen, 20 Nørre Allé, DK-2200 Copenhagen N, Denmark. Fax: +45 35 32 66 25. E-mail: ss@odont.ku.dk.

optimal bone regeneration seems limited. The purpose of the present study was to evaluate the early stages of bone healing in defects grafted with different sizes of autogenous bone particles.

MATERIALS AND METHODS

A license to perform the study was obtained from the Danish Animal Experiments Inspectorate.

Animals, Anesthesia, and Surgical Procedure

Fifteen female Copenhagen White rabbits *(Orycto-lacus cuniculus)* with closed epiphyseal plates were used. The animals were divided randomly into 3 groups of 5 animals each. The animals were kept in single cages and fed a standard dried diet (Altromin, Slangerup, Denmark) and water ad libitum.

Anesthesia was induced by fentanyl + fluanison (Hypnorm, fentanyl 0.315 mg/mL + fluanison 10 mg/mL; Janssen Pharmaceutica, Beerge, Belgium, 0.1 mL/kg IV) and midazolam (Dormicum, 5 mg/mL; Hoffmann-La Roche, Basel, Switzerland, 0.1 mL/kg IV). Dihydrostreptomycin + benzylpenicillin (Streptocillin Veterinary, dihydrostreptomycin 250 mg/mL + benzylpenicillinprocaine 200,000 IU/mL; Novo Industries, Copenhagen, Denmark, 0.25 mL/kg IM) was administered preoperatively.

The surgical field was first shaved and disinfected with iodophor and isopropyl alcohol (DuraPrep; 3M Health Care, St Paul, MN). A sagittal incision from the nasal to the occipital region was performed after local infiltration of lidocaine hydrochloride + adrenaline (Xylocain Adrenalin, lidocaine hydrochloride 20 mg/mL + adrenaline 12.5 µg/mL; Astra, Södertalje, Sweden, 1 mL). Subperiosteal dissection was carried out, and 4 bicortical bone defects with a diameter of 6 mm were prepared with a trephine (TRE 05; 3i, Palm Beach Gardens, FL) in the parietal and frontal bones under saline irrigation (Fig 1).[9] Involvement of cranial sutures was avoided. The 4 bone plugs were gently removed and a 1-mm-deep circular marking was made with a larger trephine (TRE 08; 3i) 1 mm from the edge around each defect. This marking was filled with preheated gutta-percha (Ultrafil, Hygenic Corporation, Akron, OH) for later identification of the defect edges on the histologic sections.

Three of the 4 removed bone plugs were milled by a bone mill (Roswitha Quétin bone mill; Dental-Produkte, Leimen, Germany) with 3-mm perforations to obtain small bone graft particles. The irregularly shaped particles had a size of 0.5 to 2 mm^3 estimated microscopically with a linear measure scale (Leitz, Wetzlar, Germany). The fourth bone plug was divided with a bone rongeur into 4 large bone particles with an approximate size of 10 mm^3. An expanded polytetrafluoroethylene (e-PTFE) membrane (Gore-Tex; W. L. Gore, Flagstaff, AZ) with a diameter of 8 mm was placed between the inner surface of the calvarium and dura mater.[10] Three of the 4 defects were filled at random according to the following scheme (Fig 1):

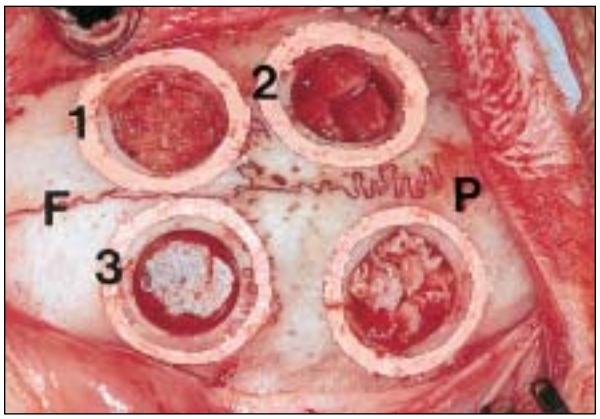


Fig 1 Experimental design. Note the e-PTFE membrane at the bottom of the control defect. F = frontal bone; P = parietal bone; 1 = defect with small particles; 2 = defect with large particles; 3 = control defect. The fourth defect was filled with Bio-Oss, but these results were later omitted from this publication.

1. Small autogenous bone graft particles
2. Large autogenous bone graft particles
3. No graft (control defect)

Defects 1 and 2 were filled with a similar weight of bone graft. All defects were soaked with autogenous blood and covered by 1 large e-PTFE membrane (GT10, Gore-Tex) fixed with 2 titanium membrane nails (Frios Fixation Set; Friatec, Mannheim, Germany). Periosteum was sutured with non-degradable sutures (Prolene 4-0; Ethicon, Norderstedt, Germany) and the skin with degradable sutures (Vicryl 4-0; Ethicon). Dihydrostreptomycin + benzylpenicillin (0.125 mL/kg IM) was administered for 3 days postoperatively. Buprenorphine hydrochloride (Anorfin, 0.3 mg/mL; GEA, Frederiksberg, Denmark, 0.17 mL/kg IM) was administered just after surgery and 2 times daily for 2 days postoperatively.

Tissue Processing

The animals were sacrificed with an overdose of sodium pentobarbital (Pentobarbital, 200 mg/mL; Den Kgl Veterinær-og Landbohøjskoles Apotek, Frederiksberg, Denmark; 0.30 mL/kg IV) 1, 2, and 4 weeks after surgery, respectively. A tissue block with all the defects and including 3 mm of surrounding

bone and attached soft tissue was fixed for 7 days in 70% ethanol.

The titanium membrane nails were removed and each tissue block was divided into smaller blocks, each containing 1 experimental defect, with a band saw (EXAKT 300 CP Band Saw System; EXAKT Apparatebau, Norderstedt, Germany). Each specimen was stored in 70% ethanol until embedding in polymethyl methacrylate resin (Bie & Berntsen, Rødovre, Denmark; and Akzo Chemicals, Deventer, The Netherlands).[11] All tissue blocks were coded by a laboratory technician to provide blinding of the following histologic and stereologic evaluation.

The sectioning has previously been described in detail in a similar study.[12] Briefly, a hard tissue microtome (Jung Microtome, Model K; Leica Instruments, Nussloch, Germany) was used to obtain 7 equidistant sections perpendicular to the bone surface throughout each defect by using a systematic random sampling design.[13,14] The sections, with a thickness of approximately 7 µm, were stained with Goldner's trichrome.[15]

Stereology

The stereologic method has also been described previously in detail in a similar study.[12] Briefly, the reference volume, *V(ref)*, defined as the volume between the 2 membranes, was estimated using the Cavalieri volume estimation principle[16]:

$$V(ref) = t \cdot a(p) \cdot \sum_{i=1}^{7} P(ref)$$

where t is the distance between the sampled sections, $a(p)$ is the area associated with each point of the used point set, and $P(ref)$ is the number of points hitting the area between the 2 membranes.

Densities (V_V) of regenerated mineralized bone, as well as remaining bone graft, were estimated by moving a systematic point set along a diagonal line from the inner membrane at one edge of the defect to the outer membrane at the other defect edge. The number of points $p(bone)$ hitting regenerated mineralized bone and bone graft, respectively, was counted. In addition, the number of points hitting the diagonal line $p(ref)$ was counted. Consequently, densities of regenerated mineralized bone as well as remaining bone graft were estimated:

$$V_V = \frac{\sum_{i=1}^{7} p(bone)}{\sum_{i=1}^{7} p(ref)}$$

Finally, total volumes of regenerated mineralized bone as well as remaining bone graft were calculated:

$$V(bone) = V_V \cdot V(ref)$$

A similar procedure using a test system of points and cycloids moved along the previously described diagonal line was performed to estimate density of vessel profiles (S_V)[14]:

$$S_V = 2 \cdot \frac{\sum_{i=1}^{7} I}{\frac{l}{p} \cdot \sum_{i=1}^{7} p(ref)}$$

where I is the number of intersections between vessel profiles and cycloids, l/p is the length of each cycloid per point, and $p(ref)$ is the number of points hitting the diagonal line. The total vessel surface area could finally be calculated:

$$S(vessel) = S_V \cdot V(ref)$$

Quantitation of 9 randomly chosen specimens (1 specimen from each group) was repeated on the originally sampled sections 2 months after the original quantitation.

Data Analysis

Data management and calculation were done using Statistical Analysis System for Personal Computers (Version 6.12; SAS Institute, Cary, NC). Data were evaluated by analysis of variance and Duncan's range test. The unit of analysis was the animal (n = 5), which was used as a classification variable in all statistical analyses, as well as treatment and length of observation period. The significance level was .05.

Sampling design was evaluated by calculating coefficient of error (CE) of reference volume, total volume of newly formed bone, total volume of remaining graft, and total vessel surface area according to the method described by Gundersen and Jensen.[13] In addition, the coefficient of variations (CV) was calculated. The repeated estimates were used to further evaluate the sampling design by calculating CEs of the differences between the first and second sets of estimates. A t test was used to test for systematic differences.

RESULTS

Two of 45 specimens, namely a 1-week and a 4-week control defect, were omitted because of technical failure during the histologic preparation.

Table 1 Summary of Histologic Observations

	1 week			2 weeks			4 weeks		
Observation	Small particles	Large particles	Control (no graft)	Small particles	Large particles	Control (no graft)	Small particles	Large particles	Control (no graft)
Defect closed by newly formed bone	No	No	No	No	No	No	Yes	No	No
Osteoid	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Woven bone	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Parallel-fibered bone	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
Lamellar bone	No	No	No	No	No	No	Yes	Yes	Yes
Remodeling, resorption, and/or apposition of graft	No	No	N/A	Yes	Yes	N/A	Yes	Yes	N/A

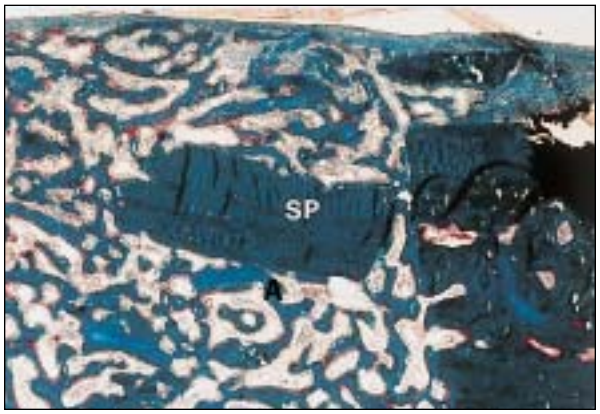


Fig 2a Defect with small particles (SP) after 2 weeks. The particle is in close contact with woven and parallel-fibered bone (Goldner's trichrome; original magnification ×23).

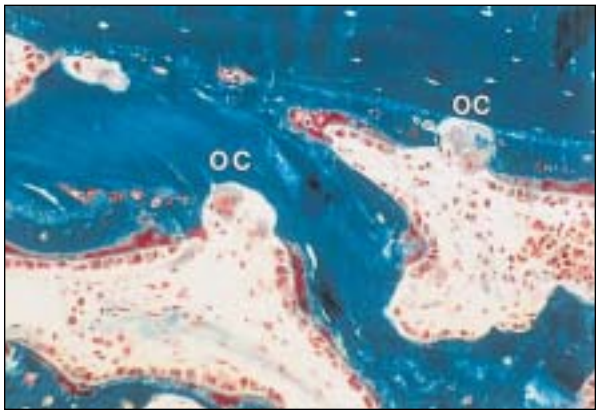


Fig 2b Higher magnification of area A in Fig 2a. Resorption of the newly formed bone by osteoclasts (OC) (Goldner's trichrome; original magnification ×152).

Histology

The main histologic observations are summarized in Table 1.

1 Week. Bone graft particles and woven bone were observed in defects filled with small as well as large particles. Woven bone extended from the edges of all defects with and without particles. Lacunae with osteocytes as well as empty lacunae were observed in particles of both sizes. Vessels were frequently noted close to the newly formed bone and sprouting toward the defect center.

2 Weeks. Although newly formed bone, mainly of a woven nature, dominated the defects with small and large particles, parallel-fibered bone was also noted. In contrast, mainly woven bone was observed in control defects. Lacunae with and without osteocytes in the grafted bone were identified. However, non-vital particles situated in a network of newly formed bone dominated defects with small particles (Figs 2a and 2b). Bone marrow was now developing in defects with small particles and could occasionally be seen communicating with the marrow of the defect edges. Bone apposition and resorption were visible in relation to both small and large particles.

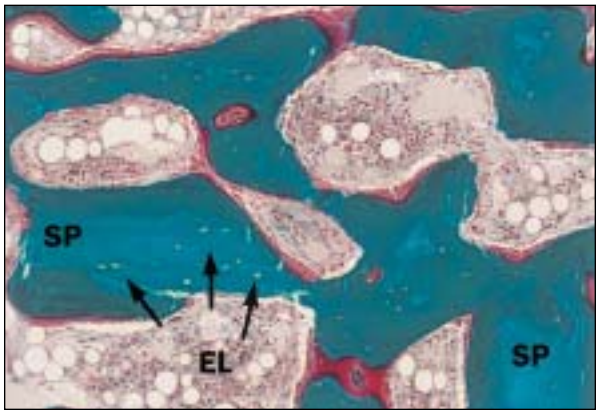


Fig 2c Defect with small particles (SP) after 4 weeks. Particles with empty osteocyte lacunae surrounded by newly formed bone (Goldner's trichrome; original magnification ×76). The empty osteocyte lacunae (EL) are indicated by arrows.

4 Weeks. Defects with small particles were completely closed by a combination of newly formed mineralized bone and well-incorporated bone graft particles (Fig 2c). The newly formed bone consisted of woven, parallel-fibered, and lamellar bone. Apposition and resorption could be noted. Osteocytes

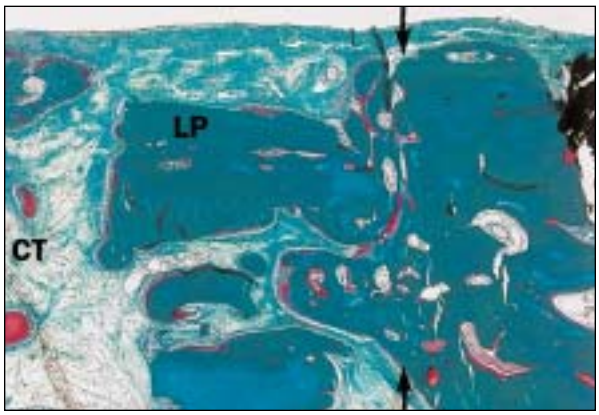


Fig 3 Defect with large particles (LP) after 4 weeks. New bone formation from the defect edge *(arrows).* Connective tissue (CT) in the center of the defect (Goldner's trichrome; original magnification ×23).

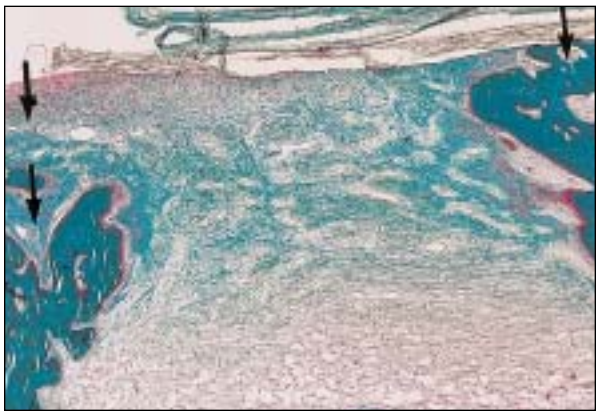


Fig 4 Control defect after 4 weeks with connective tissue in the center of the defect. Arrows indicate borders of the defect (Goldner's trichrome; original magnification ×23).

within the lacunae of the graft were rare. Redevelopment of bone marrow was seen. In addition, inner and external cortical bone plate formation was occasionally observed.

In contrast, defects with large particles were never completely closed. The gap between the particles and the defect edges was bridged by newly formed bone surrounded by vessels (Fig 3). Newly formed parallel-fibered and lamellar bone were observed, but woven bone still dominated. In the central part of the defects, fibrous connective tissue could be seen. Apposition and resorption processes dominated the bone particles. Lacunae with osteocytes in grafted bone could still be noted among the empty ones.

Incomplete healing of control defects was characteristic even after 4 weeks. All defects were dominated by woven bone extending from the edges of the defects, while connective tissue occupied the central part (Fig 4).

Stereology

The estimated reference volume of control defects was smaller compared to defects with small as well as large particles at 1, 2, and 4 weeks ($P \leq .05$) (data not shown). However, no differences were observed between defects with small and large particles ($P > .05$).

There was no difference between groups in the total volume of newly formed bone after 1 week ($P > .05$) (Fig 5). In contrast, the total volume of newly formed bone was greater in defects with small particles compared to defects with large particles and control defects after 2 and 4 weeks ($P \leq .05$).

Although no differences in total volume of remaining graft were identified between defects with small and large particles after 1 and 2 weeks ($P > .05$), the total volume of remaining graft was smaller in defects with small particles compared to defects with large particles after 4 weeks ($P \leq .05$) (Fig 6). No differences in total vessel surface area were observed between the groups after 1, 2, and 4 weeks ($P > .05$) (Fig 7).

Evaluation of Sampling Design

The CEs varied between 3% and 46%. The CEs of defects with small particles were generally smallest, irrespective of the observation length. In contrast, the CVs varied between 4% and 96%. The CEs of the differences between the 2 sets of repeated estimates varied between 0.3% and 9%. There were no systematic differences ($P > .05$).

DISCUSSION

The aim of the present study was to evaluate the initial healing events around autogenous bone graft particles of different sizes. Defects smaller than the critical-size defect (CSD)[17] were made in the calvarium of rabbits using a previously described model.[9] Investigations of the ability of bone graft materials to obtain complete bone healing of a defect necessitates use of a CSD to exclude spontaneous bony regeneration of the defect. In the present study, however, it was possible to use a smaller defect because only the early events of regeneration were investigated.

Precise identification of the former defect borders is necessary for adequate stereologic and histologic evaluation. A gutta-percha marking was therefore made around all defects. However, no difficulties were observed in identifying the former

Fig 5 Total volume of newly formed bone after 1, 2, and 4 weeks (dashes = mean).

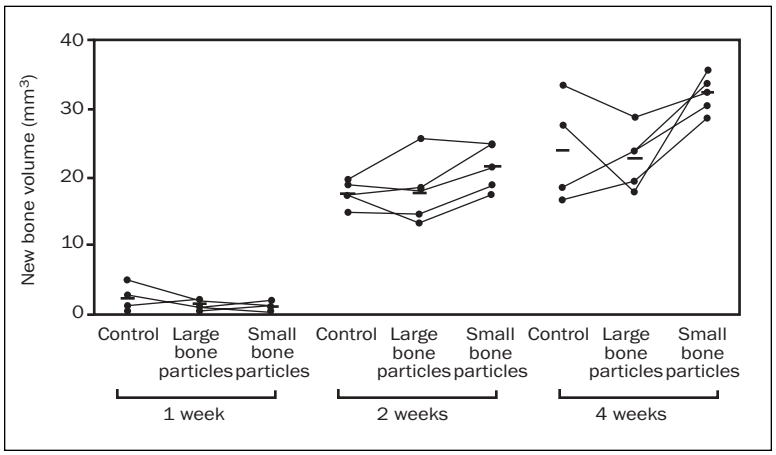


Fig 6 Total volume of remaining bone graft after 1, 2, and 4 weeks (dashes = mean).

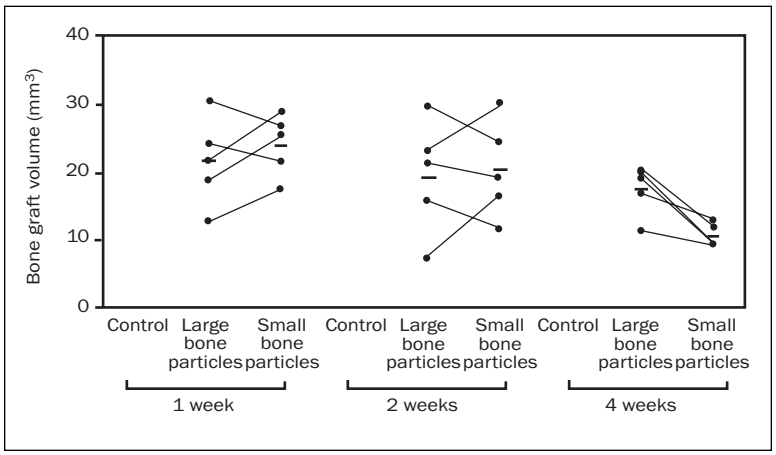
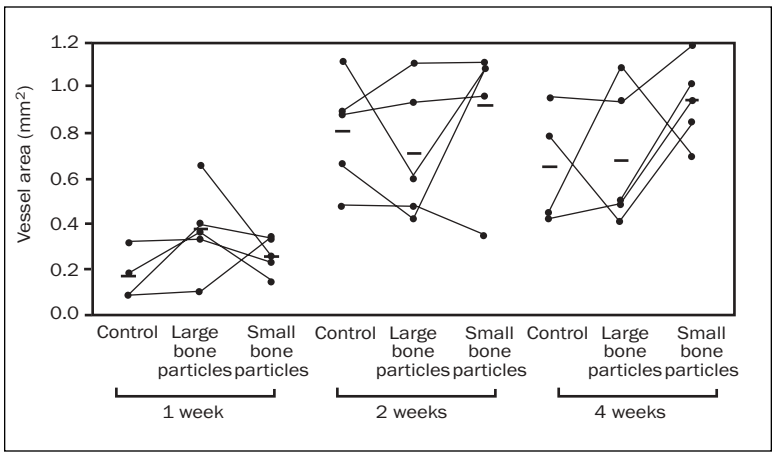


Fig 7 Total surface area of vessels after 1, 2, and 4 weeks (dashes = mean).



edges in the present study because of the short observation period. Therefore, the developed gutta-percha marking seems more useful for studies involving a longer observation period.

Healing of calvarial defects may occur from defect edges, underlying dura mater, and the overlying periosteum. However, only the regenerative capacity from defect edges and graft particles was of interest in the present study. Therefore, membranes were placed at the outer and inner surfaces of the defect to prevent disturbances from other osteogenetic sources, herniation of brain tissue, and ingrowth of connective tissue.[10,18]

No differences in diameter of the defects or surrounding bone thickness were observed between the groups (data not shown). However, the reference

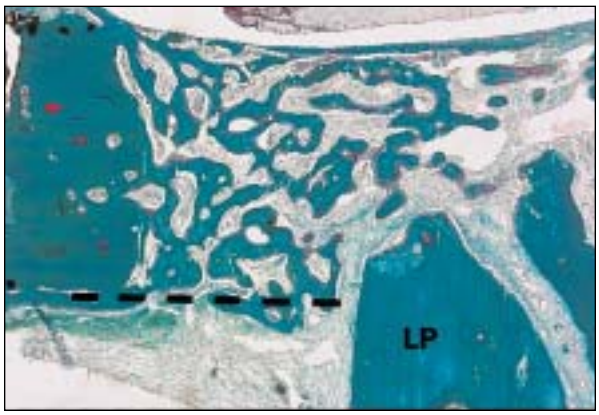


Fig 8 Defect with large particles (LP) after 2 weeks. Particles are displacing the inner membrane, enlarging the reference volume. Border of the preferred reference volume is indicated by the dotted line (Goldner's trichrome; original magnification ×23).

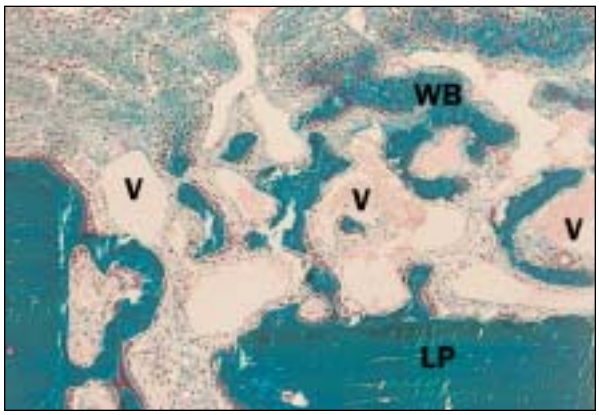


Fig 9 Defect treated with large particles (LP) after 2 weeks showing a network of vessels (V) and woven bone (WB) (Goldner's trichrome; original magnification ×58).

volume of control defects was significantly smaller than the reference volume of defects filled with small and large particles because of slight overfilling of the defects with particles that displaced predominantly the inner membrane (Fig 8). However, no significant differences were observed between defects with small and large particles.

Quantitation of total vessel surface area using stereologic methods previously has not been performed in studies of bone healing. Other methods, mainly involving histologic description, microangiography, vital staining, and postmortem injection of latex, have been used.[19–22] To facilitate vessel identification in the present study, immunohistochemical staining was considered. PAL-E, a monoclonal antibody, has been described against rabbit endothelium.[23] Unfortunately, vessel identification using this antibody was impossible in spite of extensive pilot studies. The used fixation and/or embedding procedure was not compatible with PAL-E staining. Consequently, quantitation was performed on sections stained with Goldner's trichrome (Fig 9).[15]

Although profiles of most vessels were easily recognized, the smallest vessel profiles were difficult to identify. Therefore, the amount may have been underestimated. However, lymph vessel profiles could not be distinguished from blood vessels, possibly causing slight overestimation. Also, overestimation was probably made as a result of the relatively thick sections (7 μm). Consequently, the estimated total surface area of vessels should be fairly precise, although potential bias could have been eliminated by using an antibody against the endothelium and thinner sections.[24]

The total surface area of vessels increased in all defects from weeks 1 to 2, but no differences were observed between weeks 2 and 4. This is in accordance with previous studies focusing on bone healing.[21,25] Initial bone healing is characterized by blood clot formation, neovascularization, and dilatation of adjacent vessels. Woven bone spicules begin to form at the periphery of the blood clot, where the vascularization is greatest. After defect healing with woven bone, remodeling into lamellar bone will proceed. During remodeling, no further neovascularization occurs.[25–27] This is in accordance with the present study and was especially notable in defects treated with small particles. Remodeling of the particles and the newly formed bone was already initiated between weeks 2 and 4.

A larger total surface area of vessels was expected in defects with small particles because of possible facilitated release of various growth factors and better possibilities for vessel ingrowth. However, in the present study, no differences were observed. The formation of new bone indicated that sufficient revascularization had occurred, irrespective of the particle size used.

It has been proposed that particulated autogenous bone grafts should be preferred to blocks for many bone grafting procedures.[5,8,28] Previous studies have evaluated the influence of different particle size on bone healing by using different models in humans and animals.[7,9,20,28,29] From these studies, it is impossible to conclude whether small or large bone particles should be preferred in the craniomaxillofacial region. One study using an experimental model similar to that in the present study has been performed.[9] Semiquantitation indicated increased newly formed bone in defects of rabbit calvarium filled with 0.5- to 1-mm^3 particles, compared to defects filled with bone paste, after 4 weeks. However, no differences were observed after 15 weeks.

The amount of newly formed bone in the present study was greater after 2 and 4 weeks in defects with small particles as compared to defects with large particles. In addition, resorption of small particles was more pronounced as compared to large particles after 4 weeks. These observations could be related to an increased release of growth and/or differentiation factors from the larger surface of the small particles. However, only very few isolated graft particles, surrounded partly by newly formed bone, were observed in the center of the defects in the present study. Newly formed bone replaced the graft particles by creeping substitution. Similar findings were reported in the previously mentioned study of rabbit calvarium defects.[9] This is in contrast to a previous study in dogs, which demonstrated presence of osteoinductive properties of autogenous bone grafts from the iliac crest.[28] Newly formed bone was seen arising from the grafted particles evaluated on serial microscopic sections. The background of the apparent discrepancy could be related to a difference in the animal model used and/or type of autogenous bone graft.

Unbiased stereologic methods were used within the present study.[16,30] It is important to use a sampling scheme giving a precise estimate appropriate for an optimal outcome of the investigation. The methods were evaluated by calculating the CEs and CVs. It has been recommended that the CEs are half of the CVs, as observed in the present study.[13] Consequently, the observed differences in bone regeneration using autogenous bone grafts with small and large particles seem reliable.

SUMMARY

Autogenous bone graft particles with a size of 0.5 to 2 mm^3 seem preferable to 10-mm^3 particles for bone regeneration because of the larger amount of newly formed bone around the particles, combined with more pronounced remodeling of the newly formed bone.

ACKNOWLEDGMENTS

The excellent histologic preparation by laboratory technician Rita Ullerup is gratefully acknowledged. We are also deeply indebted to Prof Dr Med H. J. G. Gundersen, Stereological Research Laboratory, Faculty of Health Sciences, University of Aarhus, for his invaluable stereologic advice. Bio-Oss and Ultrafil were kindly supplied by Geistlich Pharma, Switzerland, and Coltène Whaledent Dentalvertriebs, Germany. The study was supported by the Danish Medical Research Council, FUT-Calcin-Fondene, Direktør E. Danielsen og Hustrus Fond, and Gangstedfonden.

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