

A quantitative assessment of the healing of intramembranous and endochondral autogenous bone grafts

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SUMMARY The aim of the study was to assess quantitatively the amount of new bone formed in the early stages of healing of intramembranous and endochondral autogenous bone grafts so as to gain further insight into their integration with host bone. Eighteen critical size defects were created in the parietal bone of nine New Zealand White rabbits. In the experimental group (five rabbits), each rabbit was grafted with intramembranous bone in one defect and with endochondral bone in the other. In the control group (four rabbits), one defect was left empty (passive control) and the other was grafted with rabbit skin collagen (active control). After 14 days, the rabbits were killed and the defects were prepared for histological analysis. Serial sections were made across the whole defect. Each defect was divided into five regions spaced 1500 µm apart. Two sections were randomly drawn from each region. Quantitative analysis was performed on 100 sections using an image analyser computer software system to assess the amount of new bone formed in each defect. No bone was detected across the defect in either the active or passive controls. One-hundred-and-sixty-six per cent more new bone was formed in defects grafted with intramembranous bone than those grafted with endochondral bone. This represented an extremely significant difference ($P < 0.0001$, unpaired t -test) between the two groups. The results show that intramembranous autogenous bone produced more bone than the endochondral bone when grafted in the skull. Clinically, it is recommended that intramembranous bone is used to replace lost membranous bone in the oral cavity, as well as in skull defects, whenever possible.

Introduction

Availability of a broad, well-rounded alveolar ridge is essential for the success of orthodontic tooth movement. Cleft palate, alveolar bone defects caused by trauma or pathology, and narrow alveolar ridges are all conditions that impede the progress of orthodontic treatment. Bone grafting is essential to restore form and function to the alveolar ridge. A clinical dilemma exists regarding the type of bone that should be used. To date the choice of bone grafts in craniofacial surgery remains dependent largely on the personal preference of the surgeon. In spite of resorption, autogenous endochondral (EC) bone remains the most commonly used

graft material (Kuido and Fujioka, 1978; Burchardt, 1987). In an experimental study, Zins and Whitaker (1983) found that EC bone grafts showed a 65 per cent volume loss. Autogenous intramembranous (IM) bone grafts, on the other hand, were reported to retain volume better than EC bone grafts (Zins and Whitaker, 1983; Kusiak *et al.*, 1985; Phillips and Rahn, 1988). A possible explanation was the early and widespread revascularization of the IM bone (Zins and Whitaker, 1983; Kusiak *et al.*, 1985). In a study to examine the graft incorporation, Kusiak and co-workers (1985) reported that at day 14 there had been an average of more than 20 vessels per square for IM grafts versus 1.8

for the EC. Biomechanical and structural differences were also thought to contribute to the differences in the fate of IM and EC bone grafts (Glowacki, 1986; Phillips and Rahn, 1988, 1990). The differences in the cellular and extracellular matrices of both types of bone could also be a contributing factor to such differences (Rabie *et al.*, 1996a). EC bone grafts adopt an EC ossification route when grafted into skull defects, while IM bone grafts heal directly through bone. None of the studies referred to above have directly dealt with the issue of integration of a bone graft. An important factor that affects the integration of a bone graft is its ability to remodel and incorporate with the host bone. The incorporation of a bone graft depends on the proliferative elements in the periosteum, the endosteum, the bone marrow, and the existing osteogenic cells in the micro-environment provided by the host (Urist, 1980). This phenomenon is known as osteo-conduction. The ability of the bone graft to form new bone, osteo-induction, is a major contributing factor to the integration of the graft with the host bone (Rabie *et al.*, 1996b). Thus far, no attempt has been made to quantify the amount of new bone produced by these bone grafts, although this would provide direct evidence of the ability of a bone graft to integrate with the host bone. Therefore, the aim of this study was to examine the integration of IM and EC bone grafts to the host IM bone by assessing quantitatively the amount of new bone formed during the early stage of bone graft healing.

Materials and methods

Experimental groups

Eighteen critical size defects were created in the parietal bone of nine New Zealand White rabbits (two defects per rabbit; Figure 1). The rabbits were 5 months old (adult stage) and weighed 3.5–4.0 kg. Mature animals were chosen against immature ones as the latter can more actively repair an osseous defect (Prolo *et al.*, 1982). In the experimental group (five rabbits), each rabbit was grafted with IM bone in one defect and with EC bone in the other. In the control

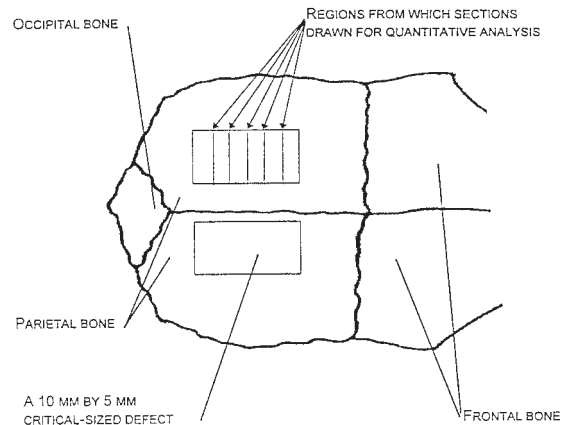


Figure 1 Schematic diagram of dorsal view of rabbit skull, showing the location of two surgically-created critical size defects and the five regions within the defect from which sections were drawn for quantitative analysis.

group (four rabbits), one defect was left empty (passive control) and the other was grafted with rabbit skin collagen (active control).

Preparation of rabbit skin collagen

The collagen was prepared from the New Zealand White rabbits' skin. The skin was cleaned with phosphate-buffered saline to remove soft tissue, minced, and treated with a series of sodium chloride solutions, distilled water, and 4 per cent acetic acid. It was then lyophilized and dried before use.

Surgical procedures

The animals were premedicated for 1 hour before surgery with oxytetracycline hydrochloride (200 mg/ml, 30 mg/kg body weight, Tetroxyla, Bimeda, Dublin, Ireland) and buprenorphine hydrochloride (0.3 ml/kg body weight, Hypnorm, Janssen Pharmaceutical, Beerse, Belgium), supplemented with diazepam (5 mg/ml, 1 mg/kg body weight, Valium 10, Roche, Switzerland). In order to maintain the level of neuroleptanalgesia, increments of Hypnorm (0.1 ml/kg) were given at 30-minute intervals during the operation.

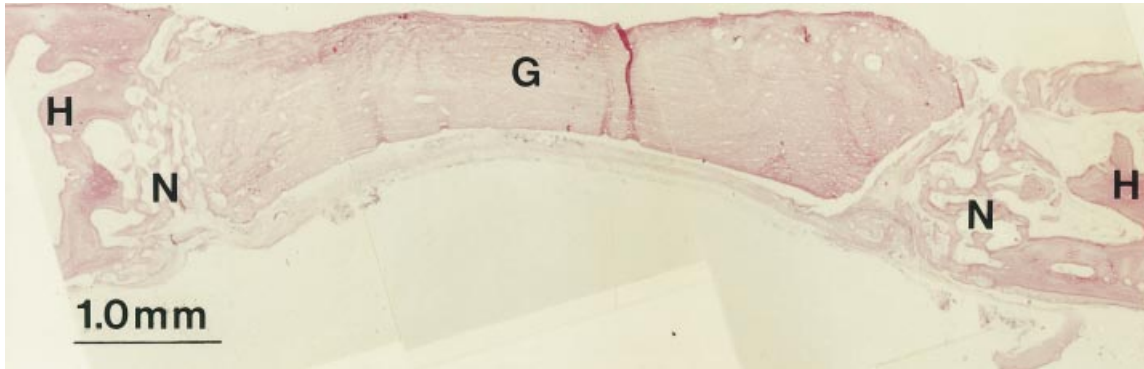


Figure 2 Photomicrograph of defect grafted with EC bone. New bone (N) is bridging from the host bone (H) to bone graft (G) (PAS, $\times 34$).

The surgical procedure consisted of the creation of two 10×5 -mm full thickness (approximately 2 mm) cranial defects, devoid of periosteum, using templates in the parietal bones (Figure 1). In the experimental groups, a piece of cranial IM bone was grafted to one defect and EC bone of an identical size harvested from the diaphyseal tibial shaft to the other defect. Holes (approximately 1 mm) were drilled at opposite ends of the bone grafts and likewise in the parietal bone to allow for fixation of the bone grafts with stainless steel wire (0.3 mm). In the control groups, the collagen used was prepared from the rabbit skin.

Post-operative care

All wounds were closed with interrupted 3/0 black silk sutures. No attempt was made to approximate the periosteum. Post-operatively, the rabbits were given oxytetracycline hydrochloride daily for 10 days and buprenorphine hydrochloride for 2 weeks.

Two weeks after surgery, the animals were killed with sodium pentobarbitone. Immediately upon death, the defects and surrounding tissue were removed for histological preparation.

Histological preparation and analysis

The tissues were fixed in 10 per cent neutral buffered formal saline solution, demineralized with K's Decal Fluid (sodium formate/formic acid,

Oral Biology Unit, University of Hong Kong), and finally double-embedded in celloidin/paraffin wax. Serial, 5 μ m thick sections of the whole defect were cut perpendicular to the long axis.

Quantitative analysis was carried out on serial sections of the defects in the experimental groups. The defects were divided into five regions spaced 1500 μ m apart (Figure 1). In each region, 10 serial sections were cut, and from these 10 sections, two were drawn at random, giving a total of 10 sections from each defect. These sections were then stained using the periodic acid-Schiff (PAS) reaction technique to show new bone and mineralizing cartilage, and cover-slipped in histological medium (Permount, Fisher Scientific, New Jersey, USA). Each section included the graft and host bone of both sides of the defect. Thus, there were two graft–host interfaces. The total amount of new bone (N; Figures 2 and 3) formed in both graft–host interfaces within the surgically-created defect was quantified by one observer (R. W.). Quantitative analysis was performed with a semi-automatic image analysis computer software system (Videoplan, Kontron Image Analysis Division, Germany), through a light transmitting microscope (Axioskop, Carl Zeiss, Germany) fitted with a video camera (Colour Video Camera, TK-1080E, JVC, Japan). Differences in staining properties and morphology between the newly formed bone, old bone, and cartilage enabled easy identification of newly formed bone.

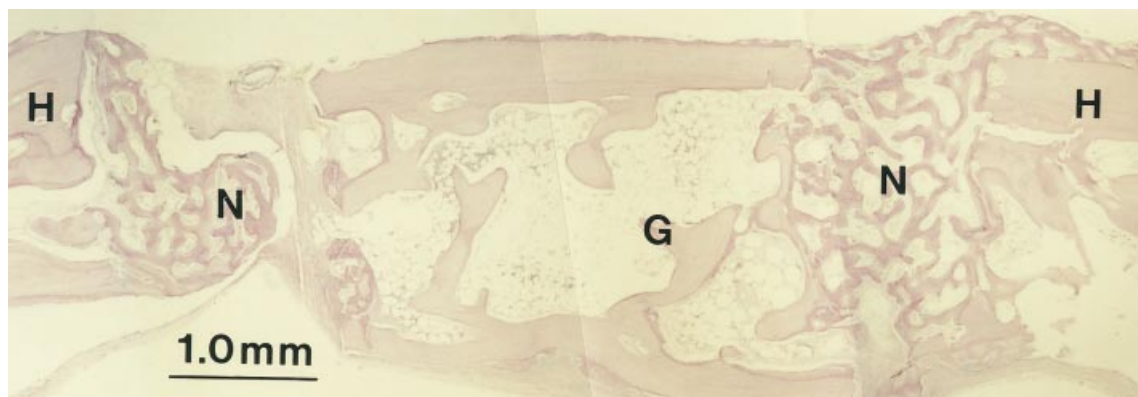


Figure 3 Photomicrograph of defect grafted with IM bone. New bone (N) is bridging from the host bone (H) to bone graft (G) (PAS, ×34).

Qualitative analysis was performed with serial sections of the defect stained with haematoxylin/phloxine B/saffron (HPS).

Statistical methods

Data were analysed using a statistical analysis computer software (Graphpad Instat, v.2.04a, 1993, San Diego, CA, USA). The one-way analysis of variance (ANOVA) method was used to compare sections drawn from the five regions in each defect. The arithmetic mean, standard deviation (SD), and 95 per cent confidence intervals were calculated for each experimental group. The two means were compared by the unpaired *t*-test, with $P < 0.05$ chosen as the critical level of statistical significance.

The size of the method error in digitizing the areas of new bone was calculated by the formula:

$$Se = \pm \sqrt{\frac{\sum d^2}{2n}}$$

where *d* is the difference between the two registrations of a pair and *n* is the number of double registrations (Dahlberg, 1940). Ten randomly drawn histological sections were digitized on two separate occasions at least 3 months apart by the same observer and also by an independent observer. Paired *t*-tests were also performed to compare the intra- and inter-observer registrations.

Results

Clinical and physical examinations

All animals remained in excellent health throughout the course of the experiment and rapidly recovered after surgery. There was no evidence of infection in any of the animals.

Macroscopic appearance

Two weeks post-operatively, the defects in the non-grafted group did not exhibit bone formation, as they were soft to palpation, whilst the defects implanted with either EC or IM bone grafts were hard to palpation.

Histological findings

Experimental groups. Histological evaluation of the healing process revealed that new bone was localized to the host bone-graft interface (Figures 2 and 3). Integration of the EC bone graft with the recipient bed was characterized by the presence of visible foci of cartilage surrounded by new bone. Integration of the IM bone graft with the recipient bed was characterized by the presence of new bone formation without cartilage.

Control groups. The defects were filled with fibrous connective tissue (Figure 4). Occasionally, a narrow margin of new bone formation

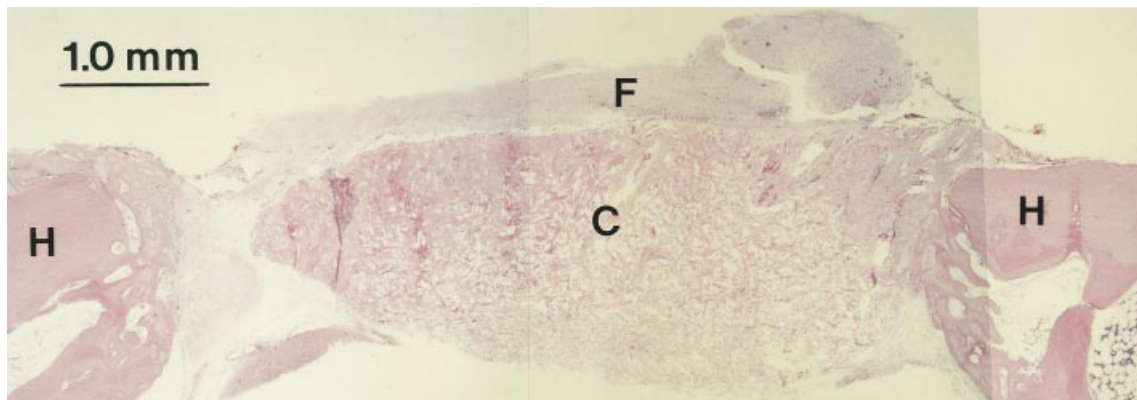


Figure 4 Photomicrograph of control, defect grafted with collagen (C). Fibrous tissue (F) is forming between the defect (H) (PAS, $\times 34$).

could be seen around the edges of the defects bridging the marrow space. The collagen grafted did not induce bone formation.

Quantitative analysis

The amount of new bone formed 2 weeks after grafting is presented in Tables 1 and 2. A total of 100 sections were digitized.

The amount of newly formed bone was significantly greater in the IM bone grafted defects than in the EC bone grafted defects. The amount of new bone formed in defects grafted

with IM bone was 166 per cent more than that formed with EC bone. An extremely significant difference ($P < 0.0001$, unpaired *t*-test, Table 3) was found between the two groups.

For method error analysis, 10 sections were drawn at random and digitized, and the readings were then compared. The method error of the image analysis did not exceed 0.014 mm^2 which was insignificant compared with the results. Hypothesis testing indicated no significant difference among the duplicate intra-observer ($P = 0.565$) and inter-observer registrations ($P = 0.591$) of the 10 randomly drawn sections.

Table 1 Summary of amount of new bone (mm^2) in five defects grafted with EC bone. Mean, standard deviation (SD), and results of ANOVA [degrees of freedom (df), *F*, *P*], comparing five regions within each defect.

Region	Defect I	Defect II	Defect III	Defect IV	Defect V
A	0.56	0.36	0.63	0.51	1.03
	0.57	0.36	0.64	0.40	1.03
B	0.77	0.46	0.51	0.56	1.15
	0.67	0.46	0.50	0.56	1.07
C	0.63	0.33	0.52	0.48	0.92
	0.58	0.34	0.53	0.48	0.91
D	0.66	0.42	0.57	0.46	1.01
	0.66	0.38	0.59	0.46	1.04
E	0.53	0.47	0.51	0.89	0.99
	0.53	0.55	0.52	0.97	0.95
Mean	0.616	0.413	0.552	0.577	1.01
SD	0.076	0.071	0.052	0.016	0.07
df	4,5	4,5	4,5	4,5	4,5
<i>F</i>	9.075	12.765	74.875	44.022	11.806
<i>P</i>	0.016	0.008	<0.001	<0.001	0.009

Table 2 Summary of amount of new bone (mm²) in five defects grafted with IM bone. Mean, standard deviation (SD), and results of ANOVA [degrees of freedom (df), *F*, *P*], comparing five regions within each defect.

Region	Defect I	Defect II	Defect III	Defect IV	Defect V
A	1.70	1.40	1.62	1.76	1.77
	1.95	1.45	1.71	1.75	1.67
B	1.57	1.59	2.06	1.77	1.59
	1.44	1.74	2.07	1.78	1.56
C	1.42	1.24	2.10	1.63	1.99
	1.37	1.26	2.21	1.67	1.95
D	1.80	1.52	1.77	1.63	1.90
	1.80	1.45	2.06	1.58	1.86
E	1.29	1.40	1.80	1.71	1.91
	1.30	1.38	1.81	1.70	1.92
Mean	1.564	1.443	1.921	1.698	1.812
SD	0.235	0.148	0.200	0.069	0.155
df	4,5	4,5	4,5	4,5	4,5
<i>F</i>	13.916	14.891	7.393	22.932	36.912
<i>P</i>	0.006	0.006	0.025	0.002	<0.001

Table 3 Comparison of area of newly formed bone between defects of EC and IM bone grafts (mm²).

	Mean	SD	Mean difference	<i>P</i>
EC group	0.634	0.226		
IM group	1.687	0.238	1.053	<0.0001

Discussion

The rabbit model used in this study was relevant because non-grafted control-critical size defects have been found not to heal spontaneously during the life of the animal (Schmitz and Hollinger, 1986). The defect was 10 × 5 mm and of full thickness of the parietal bone, and the periosteum was totally disrupted and not replaced. A thin film of new bone produced by the host tissues, through osteo-conduction, could be seen adjacent to the host bone. However, there was no bone healing across the defect with or without collagen grafts as shown by the results of the control group (Figure 4). In a previous study, the route of healing of EC and IM bone grafts was examined in a similar model (Rabie *et al.*, 1996a). Cartilage was detected on day 5 and endochondral ossification

concomitant with vascular invasion was seen on day 7. By day 14, bone remodelling was evident in both IM and EC bone grafts. Therefore, in this investigation it was decided to assess quantitatively the amount of new bone formed on day 14 which gives a better indication of the ability of a bone graft to integrate with the host bone during the early stages of healing. Later stages of bone graft healing have been the subject of earlier studies. Zins and Whitaker (1983) examined the volume maintenance of IM and EC bone grafted on the snout of rabbits, and the volume of bone grafts was compared to their volume when retrieved after 5, 10, and 20 weeks. The IM bone maintained its volume to a significantly greater extent than EC bone. Smith and Abramson (1974) examined healing of bone grafts up to 12 months, and also demonstrated that IM bone underwent less resorption than EC bone.

The objectivity of this research is enhanced by three factors:

- (1) the extensive and detailed design of the serial sectioning to represent new bone formation in the defect (Figure 1);
- (2) quantitative analysis by computer-assisted image analysis (Eppley *et al.*, 1991);

- (3) extensive statistical methods that compared different regions in each defect, the difference in the amount of new bone formed between study groups, and the method error analysis in digitizing the areas of new bone.

The results of the ANOVA of the different regions within each defect showed the necessity to analyse multiple regions within each defect (Tables 1 and 2). This was indicated by (the P value) the difference in the amount of new bone formed between the different regions within each defect being statistically significant.

The method utilized in measuring the amount of new bone was reliable, as indicated by the fact that the difference between two paired intra-observer readings was not statistically significant ($P = 0.565$) and the small size of the method error. The difference between two paired inter-observer readings was not statistically significant ($P = 0.591$), this indicated that the systemic error caused by the observer was small.

The present study demonstrated that IM bone produced 166 per cent more new bone than EC bone when grafted into skull defects. One may question whether it was the difference in the proportions of cortical and cancellous bone between the EC and the IM grafts that caused the difference in the new bone formation observed in this study. While the EC bone grafts used in this study may have contained more cortical bone and may, however, take longer to re-vascularize, earlier studies have shown otherwise. In an investigation by Kusiak and co-workers (1985), the IM bone re-vascularized more rapidly than the EC bone despite the fact that the EC bone taken from the iliac crest was more cancellous than the IM bone. Therefore, it is conceivable that the differences in the re-vascularization pattern of IM and EC bone graft are more dependent on the nature of the extracellular matrices, and their content of angiogenic mediators and other growth factors than on the three-dimensional osseous architecture of the grafted bone. In addition, Hardesty and Marsh (1990) studied the influences of the number of graft cortices (cortical or bicortical) and graft orientation (graft cortex to bed relationship) on the healing of both EC and IM grafts. The IM onlay

grafts maintained or even increased their volume, surface area, and weight, whereas the EC onlay grafts lost volume, surface area, and weight. Neither the number of graft cortices nor graft orientation affected the graft fate.

The results of this study introduce an additional explanation to the enthusiasm shown towards IM bone grafts in earlier clinical (Phillips and Rahn, 1988) and experimental (Smith and Abramson, 1974; Zins and Whitaker, 1983; Kusiak *et al.*, 1985) investigations. These reports advocated the use of IM onlay bone grafts over EC bone because of the former's ability to maintain volume (Zins and Whitaker, 1983; Kusiak *et al.*, 1985; Phillips and Rahn, 1988). Volume maintenance was thought to be due to the fact that IM bone grafts underwent less resorption than EC bone grafts (Zins and Whitaker, 1983). The current data suggest that volume maintenance of IM bone grafts could also be due to the ability of IM bone to integrate and incorporate with the host calvarial bone. Graft incorporation is dependent on many inter-related processes including osteo-progenitor cell proliferation, osteoblast differentiation, osteo-induction, osteo-conduction, and the biochemical nature of the extracellular matrix of bone graft. Integration of IM bone grafts with the recipient's calvarial bone is characterized by the presence of osteogenic cells without passing through a cartilage intermediate stage (Rabie *et al.*, 1996a). When EC bone is grafted into calvarial bone defects, the integration is characterized by the presence of visible foci of cartilage surrounded by new bone (Rabie *et al.*, 1996a). The cells involved in the repair of IM and EC bone, as well as the origin of the recipient bone (IM or EC) play an important role in the incorporation and integration of a graft. Strawich and Glimcher (1983) carefully compared histological, histochemical and biochemical parameters of chick long bones and cranial bones at various stages of development, and found differences in the post-translational modifications of collagens of both types of bone, thus highlighting some differences between osteoblasts of different osseous tissues. Similar results were reported by Moskalewski and co-workers (1988), who also suggested that osteoblasts from EC scapular bone have different

properties from those of IM calvarial bone. Furthermore, an osteo-inductive factor has been extracted from IM bone which is different from that of EC bone (Scott *et al.*, 1994). A heparin sepharose-binding, osteo-inductive factor, extracted from bovine IM bone matrix and partially purified, exhibited a different molecular mass than that of a comparable osteo-inductive factor isolated from EC bone. Therefore, the lack of volume maintenance of EC bone grafted into host IM bone in origin could be due to lack of integration and incorporation. Similarly, the volume maintenance of IM bone when grafted into an IM defect could be due to the improved integration and amalgamation of the graft with the host bed. The current data support a sound surgical practice that proposes to replace same for same. Therefore, in defects in the oral cavity such as alveolar clefts, cleft palate, or narrow alveolar ridge, it would be more appropriate to use bone grafts of IM origin whenever possible.

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