

Effect of Bio-Oss[®] Collagen and Collagen Matrix on Bone Formation

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Abstract: *Objective:* to compare the amount of new bone produced by Bio-Oss[®] Collagen to that produced by collagen matrix *in vivo*.

Method: eighteen bone defects, 5mm by 10mm were created in the parietal bone of 9 New Zealand White rabbits. 6 defects were grafted with Bio-Oss[®] Collagen. 6 defects were grafted with collagen matrix alone (positive control) and 6 were left empty (negative control). Animals were killed on day 14 and the defects were dissected and prepared for histological assessment. Quantitative analysis of new bone formation was made on 100 sections (50 sections for each group) using image analysis.

Results: A total of 339% more new bone was present in defects grafted with Bio-Oss[®] Collagen than those grafted with collagen matrix (positive control). No bone was formed in the negative control group.

Conclusion: Bio-Oss[®] Collagen has the effect of stimulating new bone formation locally compared with collagen matrix *in vivo*. Bio-Oss[®] Collagen may be utilized as a bone graft material.

Keywords: Bone graft, Collagen, Bio-Oss[®] Collagen.

INTRODUCTION

Discovery of bone substitute that enhance bone formation or to improve bone healing is needed for the treatment of bone trauma or surgery. The use of autogenous bone grafts requires a harvesting procedure at a donor site, which means increased morbidity [1,2]. In addition, graft resorption is a problem. Zin and Whitaker showed autogenous bone graft have 65% of bone loss after grafting [3]. One way to overcome this problem would be to use bone substitutes alone as a osteoconductive scaffold for bone regeneration from the residual bone [4] or in combination with autogenous bone, which also has osteoinductive properties [5]. Early studies with xenografts like the 'Kiel bone' or 'Oswestry bone' half a century ago showed side effects like sterile fistulas and their use was discontinued [6-8]. Bio-Oss[®] (Geistlich Pharmaceutical, Wolhusen, Switzerland) is deproteinized bovine cancellous bone developed in the last decade with a structure similar to human bone and with osteoconductive properties [9]. It is a natural, non-antigenic, porous bone mineral matrix. It is produced by removal of all organic components from bovine bone. Several animal studies have shown this material to be promising in comparison with other bone substitutes [9,10]. Bio-Oss[®] Collagen is a combination of purified cancellous natural bone mineral granules (Bio-Oss[®]) and 10% collagen fibres in a block form and is sterilized by γ -irradiation. The collagen facilitates handling of the graft particles and acts to hold the Bio-Oss[®] Collagen at the desired place. The consistency of this material readily allows it to take the shape of the defect. Clinically it does not seem to have the immunological side

effects mentioned above [11,12]. It is of interest to know whether it enhances the healing of a bone defect by increasing the amount of new bone formation locally. Therefore, in the present study we examined the amount of new bone produced by Bio-Oss[®] Collagen grafted into bony defects in rabbits and compared with those of collagen matrix graft (positive control) and no graft (negative control). Collagen matrix (purified absorbable fibrillar collagen) was used in this study because it had been used successfully as a carrier for growth factors like BMP-2 to induce bone formation in animals and in humans [13]. It was derived from bovine tendon, in the fibrillar form and was suggested from the Manufacturer (Collagen Matrix, Inc NJ, USA) to be useful for delivering cells and growth factors and for gene therapy and it was successfully used with rhBMP-2 in the repair of alveolar clefts in humans [14].

MATERIALS AND METHODS

Preparation of Bio-Oss[®] Collagen (Experimental Group)

0.2 mL of water of injection was mixed with 100mg of Bio-Oss[®] Collagen to form a paste. The mixture was soaked for 15 minutes before grafting.

Preparation of Collagen Matrix (Positive Control Group)

0.2 mL of water of injection was mixed with 20mg of collagen matrix (purified fibrillar collagen, Collagen Matrix, Inc NJ, USA) to form a paste. The mixture was soaked for 15 minutes before grafting.

Experimental and Control Groups

The protocol of Wong and Rabie [15] was followed. Eighteen 10×5mm full-thickness bone defects were created in the parietal bones of 9 New Zealand White rabbits from an inbred colony. The rabbits were 5 months old (adult

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stage) and weighed 3.5-4.0 kg. The handling of the animals and the experimental protocol were approved by the Committee on the Use of Live Animals in Teaching and Research, the University of Hong Kong. In the experimental group, 6 defects were grafted with Bio-Oss® Collagen. In the control groups, 6 defects were grafted with collagen matrix (positive control); 6 defects were left empty (negative control). The sample size was based on previous research using this model. For the experimental group and the control groups, two defects were created on the parietal bone of each rabbit. In each group six defects were created and surgery performed but after sacrifice only five was randomly drawn and prepared for analyses.

Surgical Procedures

The animals were premedicated 1 hour before surgery with oxytetracycline hydrochloride (200mg/mL, 30mg/kg body weight, Tetroxyla, Bimeda, Dublin, Ireland) and buprenorphine hydrochloride (0.3mL/kg body weight, Hypnorm, Janssen Pharmaceutical, Beerse, Belgium), supplemented with diazepam (5mg/mL, 1mg/kg body weight, Valium 10, Roche). In order to maintain the level of neuroleptanalgesia, increments of Hypnorm (0.1mL/kg) were given at 30-min intervals during the operation. The surgical procedure consisted of the creation of one or two 10×5mm full-thickness (approximately 2mm) cranial defects, devoid of periosteum, using templates, in the parietal bones (Fig. 1). The defects were produced using round stainless steel burs (1mm in diameter) on a low speed dental drill. Outlines of the defects were made initially by making holes of full thickness the parietal bone using a stainless steel wire template bent to the required size of the defect. The holes were joined to complete the process. During the cutting of bone, copious amount of sterile saline was used for irrigation and to minimize thermal damage to the tissues. Depending on which groups in which the rabbit belonged, the defects were grafted with different materials. In the experimental groups, the defects were filled with Bio-Oss® Collagen or collagen matrix as described above. In the negative control group, the defects were left alone.

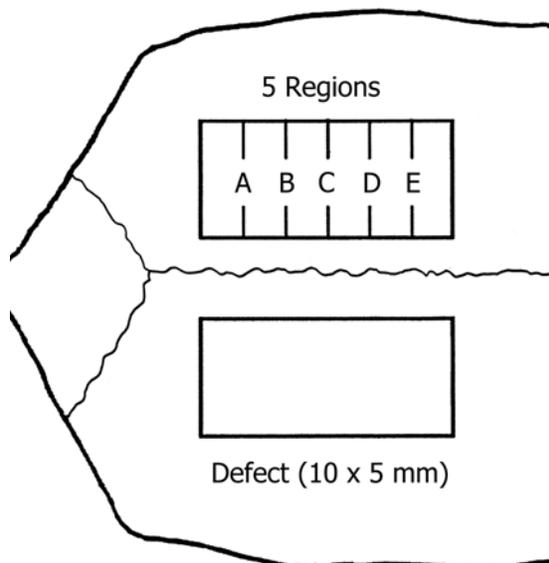


Fig. (1). diagram of the dorsal view of the skull of a rabbit, showing the sites of two surgically-created bone defects.

Postoperative Care

All wounds were closed with interrupted 3/0 black silk sutures. No attempt was made to approximate the periosteum to prevent the barrier effect. Postoperatively, 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, defects and surrounding tissue were removed for histological preparation.

Histological Preparation and Analysis

Tissues were fixed in 10% neutral buffered formal saline solution, demineralized with K's Decal Fluid (sodium formate/ formic acid), and finally double embedded in celloidin/ paraffin wax. Each tissue sample containing the defect was embedded intact. Serial, 5- μ m-thick sections of the whole defect were cut perpendicular to the long axis. Quantitative analysis was made on serial sections of defects in the experimental and the active control groups. Defects were divided into 5 regions spaced 1500 μ m apart (Fig. 1). From the serial sections in each region, 2 sections were selected randomly, giving a total of 10 sections from each defect. Therefore, the amount of new bone formation was assessed throughout the whole defect. The total amount of new bone formed within the surgically-created defect was measured on 100 sections with a technique previously described Wong and Rabie [15,16]. Each section included the graft and host bone of both sides of the defect. Thus there were 2 graft-host interfaces. The total amount of new bone formed in both graft-host interfaces within the surgically created defect was quantified by one observer (blinded - who did not know which group he was measuring), by outlining the periphery of the newly formed bone image in the computer screen (Leica Qwin Image Processing & Analysis Software, V2.3, Leica Microsystems Imaging Solutions Ltd, UK) using a transmitted light microscope (Leica, DMLB, Germany) fitted with a video camera (Single CCD Color Camera, Tk-C1380E, JVC, Japan). Differences in staining properties and morphology between newly-formed bone and mature bone made identification easy (Fig. 2). The computer image analysis system then calculated the area of the outlined new bone.

Statistical Methods

Data were analyzed using a statistical analysis software (Graphpad Instat, v.2.04a, 1993, San Diego, 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 means (Bio-Oss® group and collagen matrix positive control group) were compared by the Welch's unpaired *t*-test which does not assume equal variances, 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 $\pm \sqrt{\frac{\sum d^2}{2n}}$, where *d* was the difference between the two registrations of a pair and *n* was the number of double registrations. The size of the

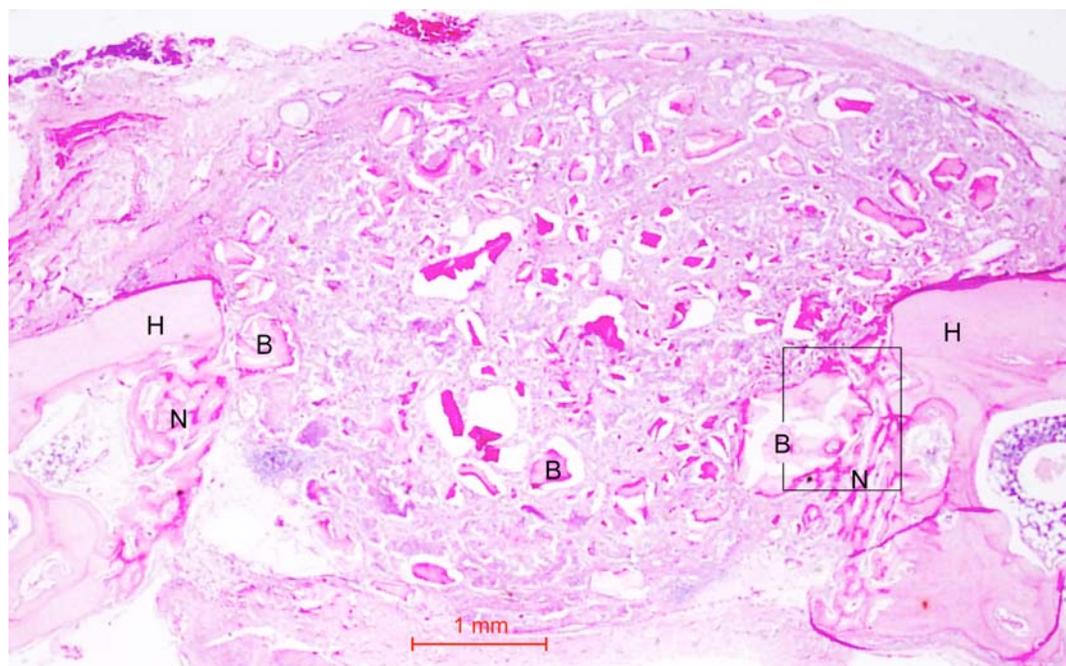


Fig. (2). photomicrograph showing healing of a bony defect grafted with Bio-Oss® Collagen on day 14. New bone (N) can be seen near the margins of the defect. H = host bone. Some Bio-Oss® granules (B) remained at the centre of the bony defect (periodic acid-Schiff stain, original magnification $\times 40$). Image inside box was enlarged in Fig. (3).

method error is 0.014mm^2 . Ten randomly drawn histological sections were digitized on two separate occasions at least three months apart by the same observer and also by an independent observer. Paired *t*-tests were also performed to compare the intra-observer and the inter-observer registrations. The two-tailed *p* value to compare the intra-observer registrations was 0.5652, that to compare the inter-observer registrations was 0.5911, both considered not significant.

RESULTS

Clinical and Physical Examinations

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

Histological Findings: Experimental Group

In the group grafted with Bio-Oss® Collagen, new bone was formed at the host bone-graft interface and tended to grow across the defect (Fig. 2). Integration of the Bio-Oss® Collagen with the recipient bed was characterized by the presence of new bone. No cartilage was found. At higher magnification (Fig. 3), new bone could be seen growing towards and amalgamating with the Bio-Oss® granules, bone cells were present showing that the collagen was not just calcified, rather, new bone was formed. In the collagen matrix positive control group little new bone was formed at the host bone – graft interface. Some collagen fibers were present at the centre of the defects (Fig. 4). In the negative control group, the defect was healed, with fibrous tissue bridging across the defect. Very little new bone had formed at the ends of the host bone, therefore no quantitative analysis was performed.

Quantitative Analysis

A total of 100 sections of the experimental group and positive control group were digitized and analyzed. The amount of newly formed bone was significantly greater in the defects grafted with Bio-Oss® Collagen than in those grafted with collagen matrix (positive control) (Tables 1-3). In the Bio-Oss® Collagen group, 50 sections were measured; the mean area of newly formed bone in each defect was 1.80mm^2 , with a standard deviation of 0.75mm^2 . In the positive control group, 50 sections were measured; the mean area of newly formed bone in each defect was 0.41mm^2 , with a standard deviation of 0.27mm^2 . Welch's unpaired *t* test which does not assume equal variances was used to test the difference between the two groups, the two-tailed *p* value is <0.0001 , considered significant.

DISCUSSION

In vivo Bio-Oss® Collagen has significantly more new bone formation locally compared with collagen matrix (positive control) when grafted into skull defects. It produced 339% more new bone than the collagen matrix, (Table 3). The difference was significant ($p < 0.0001$, unpaired *t* test). This showed that Bio-Oss® Collagen was more osteogenic when compared with collagen matrix.

The rabbit model used in this study was relevant because non-grafted negative control bone defects have been found not to heal with new bone formation within fourteen days after their creation. In addition, there was minimal bone healing across the defect as shown by the results of the negative control group. There was minimal morbidity due to this procedure as all the rabbits were in good health and condition after the surgery. Two weeks was chosen to examine the bone formation during the early healing of the bone defect was based on another study [16]. It gave better

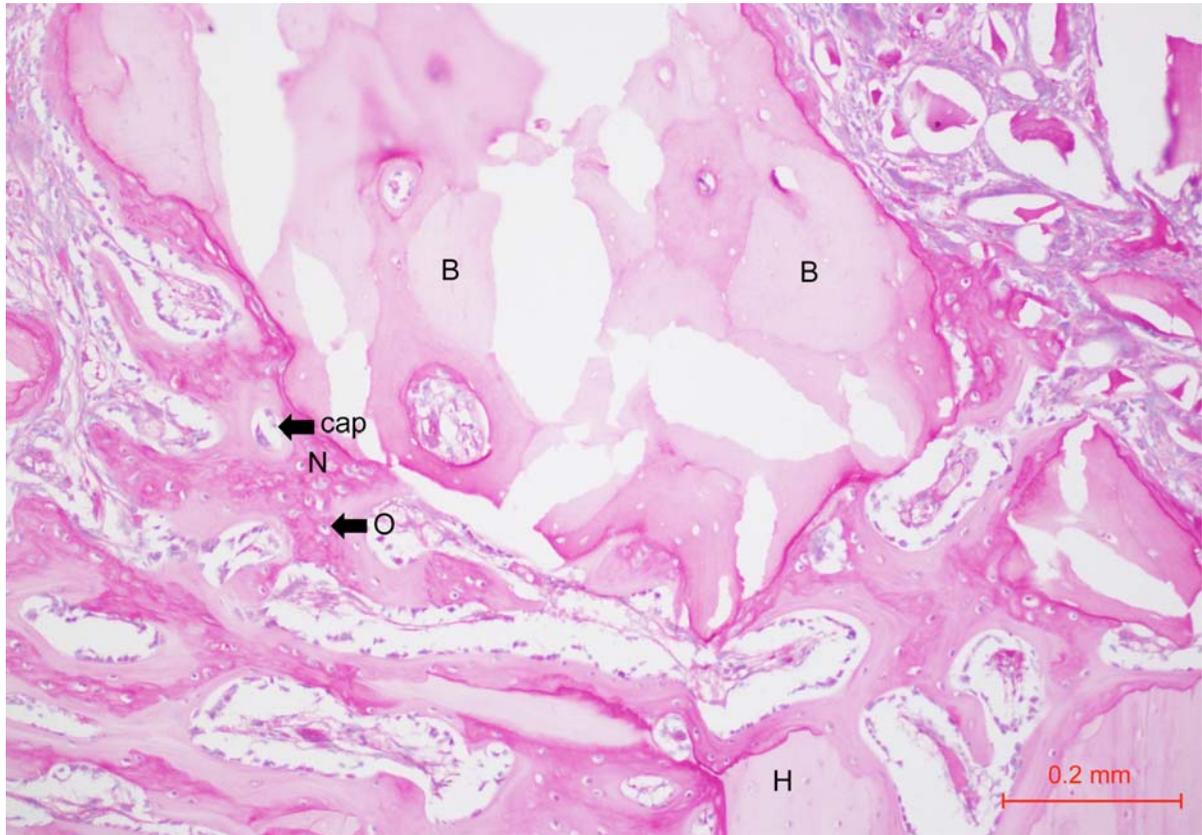


Fig. (3). high power photomicrograph showing the formation of new bone in a bony defect grafted with Bio-Oss® Collagen (box in Fig. 2). New bone (N) with osteocytes (O) could be seen growing around the Bio-Oss® granules (B) from the margin of the host bone defect (H). Capillaries (cap) were present. No cartilage was found. (periodic acid-Schiff stain, original magnification $\times 200$).

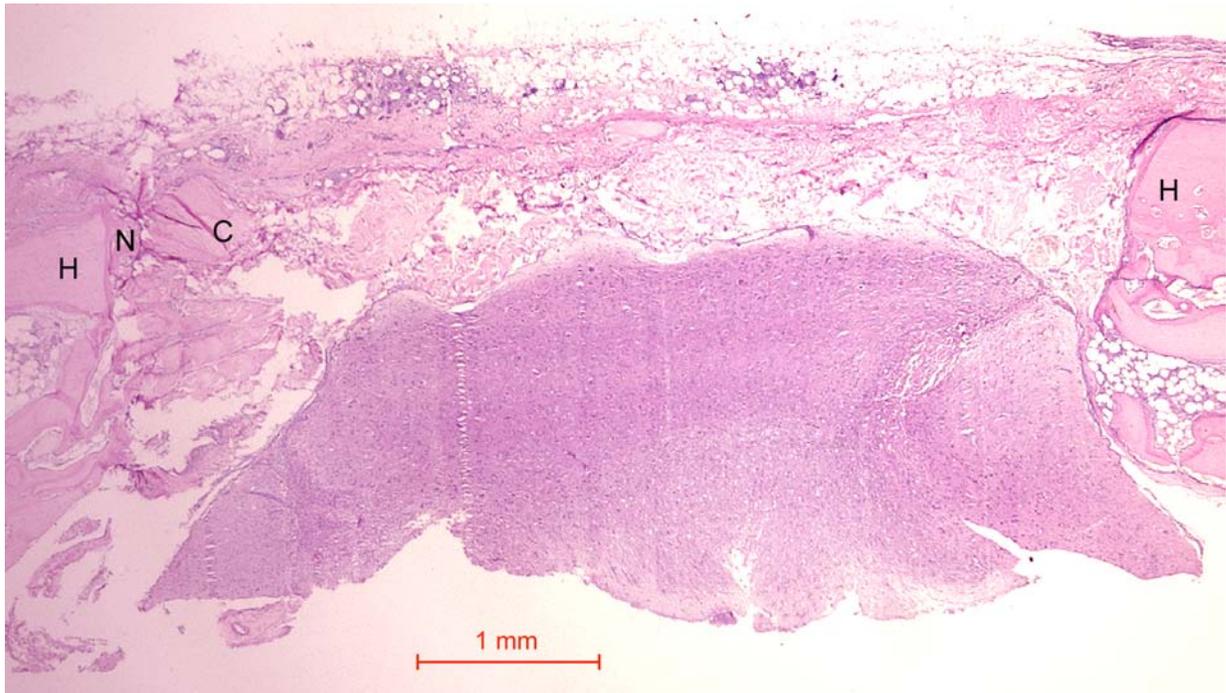


Fig. (4). photomicrograph of defect grafted with collagen matrix in day 14. No bone could be seen across the defect except for a little new bone (N) near the ends of the host bone (H). Collagen matrix (C) remained across the bone defect (periodic acid-Schiff stain, original magnification $\times 40$).

Table 1. Comparison of Amounts of New Bone (mm²) in Five Defects Grafted with Bio-Oss® Collagen. Mean and Standard Deviation (SD) of the 10 Sections of Each Defects, and Results of ANOVA (Degree of Freedom [df], F, P) Comparing Five Regions within Each Defect

Region	Defect 1	Defect 2	Defect 3	Defect 4	Defect 5
A	2.72	1.22	1.56	2.13	3.47
	2.70	1.24	1.50	2.25	3.18
B	1.96	1.23	1.52	1.91	3.86
	1.97	1.06	1.37	1.05	3.28
C	1.47	0.87	1.77	1.23	1.99
	1.54	0.89	2.23	0.96	1.72
D	1.29	1.30	1.75	1.40	2.80
	1.80	0.89	1.65	1.11	3.02
E	1.66	1.52	1.12	1.59	2.71
	1.67	1.01	1.43	1.47	3.13
Mean	1.878	1.123	1.59	1.51	2.916
SD	0.4857	0.2142	0.2944	0.4552	0.6498
df	4,5	4,5	4,5	4,5	4,5
F	18.735	1.004	4.422	3.787	11.980
P	0.0033	0.4841	0.0672	0.0883	0.0090

Table 2. Comparison of Amounts of New Bone (mm²) in Five Defects Grafted with Collagen Matrix Positive Control. Mean and Standard Deviation (SD) of the 10 Sections of Each Defects, and Results of ANOVA (Degree of Freedom [df], F, P) Comparing Five Regions within Each Defect

Region	Defect 1	Defect 2	Defect 3	Defect 4	Defect 5
A	0.68	0.64	0.18	0.12	0.41
	0.74	0.82	0.18	0.11	0.42
B	0.57	0.68	0.15	0.31	0.29
	0.51	0.71	0.16	0.38	0.34
C	0.70	0.85	0.35	0.23	0.31
	0.70	0.96	0.32	0.18	0.35
D	0.58	0.86	0.20	0.22	0.07
	0.74	0.95	0.21	0.24	0.08
E	0.60	0.51	0.06	0.15	0.11
	0.80	0.62	0.06	0.13	0.09
Mean	0.662	0.76	0.187	0.145	0.247
SD	0.09223	0.1503	0.09393	0.07412	0.1431
df	4,5	4,5	4,5	4,5	4,5
F	1.379	6.494	179.23	34.071	96.734
P	0.3603	0.0324	<0.0001	0.0008	<0.0001

Table 3. Comparison of Mean (SD) Area of Newly Formed Bone (mm²) Between Defects Grafted with Bio-Oss[®] Collagen and that of Defects Grafted with Collagen Matrix (mm²)

Group	Mean (SD)	P value
Bio-Oss [®]	1.80 (0.75)	< 0.0001
Positive Control	0.41 (0.27)	

indication of the ability of new bone to grow across the bone defect. It was also the time span chosen for other studies on bone formation using the same animal model [15-22] so that comparisons can be performed.

The results of the ANOVA of the different regions within each defect showed the necessity to analyze multiple regions within each defect (Tables 1, 2). This was indicated by the *p* value, the difference in the amount of new bone formed between the different regions within each defect was statistically significant in many defects. There was variability because the two sections in each region to be measured were randomly selected among the serial sections. Despite the variations in the amount of bone formation in different regions and in different defects, the amount of bone formation of the Bio-Oss[®] group was greater than that in the collagen matrix positive control group.

This is the first study that demonstrated Bio-Oss[®] Collagen has significant local osteogenic effect. It is possible that the surfaces of the Bio-Oss[®] granules facilitate mineralization as new bone was forming around the granules (Fig. 3). This may provide a surface for mineralization or provide a source of calcium ions which facilitate mineralization. Further research is needed to gain further understanding on its bone forming mechanism and to optimize its use and to demonstrate that Bio-Oss[®] granules are lack on antigenicity and do not show long term side effects.

To conclude, Bio-Oss[®] Collagen has effect of increasing new bone formation locally and this may be utilized for bone grafting.

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