Effects of a New Allograft Processing Procedure on Graft Healing in a Canine Model

A Preliminary Study

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Abstract Graft healing in vivo can be affected by allograft processing. We asked whether a new processing technique influenced graft-host healing compared with autograft and a standard processing technique in a canine ulna model. We used bilateral intercalary allografts or autografts in the ulna of 13 skeletally mature male coonhounds. Each animal received two allografts, either one autograft and one allograft, or two autografts. At term (90 days), the graft sites were harvested. We assessed union with high-resolution xray imaging. Each specimen was processed for nondecalcified histologic analysis to assess the graft-host interface. Quantitative histomorphometric

analysis was performed to determine spatial location and area of bone. Radiographic analysis, histologic analysis, and histomorphometric measures revealed no differences in union, mean total bone area, or total endosteal/intramedullary bone for the new process, standard process, and autografts. Our preliminary data suggest the new processing techniques may increase the safety of allograft transplantation without adversely affecting union when compared with standard processing techniques and autograft in a canine model.

Introduction

Bone allografts are a vital option for skeletal insufficiency in trauma, joint reconstruction, musculoskeletal tumors, or other reconstructive procedures. More than 986,000 bone allografts were distributed in the USA in 2002, and their usage is growing [2]. In 2002, the Musculoskeletal Transplant foundation (MTF) distributed approximately 250,000 bone grafts.

The clinical outcome of bone grafting depends mainly on the graft, fixation, and the host site [9]. Autograft is considered the gold standard to which other grafts are compared, and the healing ability and remodeling of allografts normally are less when compared with autografts [10, 11, 24, 28, 29, 33, 37, 59]. Bone allografts have the potential for disease transmission, although reported cases are uncommon [1, 15]. Allograft use, regardless of appropriate grafting procedures, intermittently results in deficient healing, perhaps caused by immunologic reactions that interfere with the healing process [9, 12, 13, 49]. There is global use of allograft bone, but there are unanswered issues related to allograft healing, remodeling, and immunology [5, 26].

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Cortical bone allografts are processed and cleaned before use for long-term preservation, to reduce the potential for disease transmission, and to reduce immunogenicity [2, 8, 17, 21, 23, 25, 27, 32, 38–40, 44–46, 52–56, 58]. In addition, there is a delicate balance between cleaning a graft and maintaining its physical and biologic properties. Chemical methods used to clean grafts include solutions of saline [21], antibiotics [8, 32, 44, 46, 52, 58], detergents [44, 51], alkylating agents [3], halogens [44], peroxides [20, 51], organic solvents [44], acids [3, 23, 40, 55], alcohols [3, 23, 25, 32, 40, 44, 46, 51, 56], and supercritical carbon dioxide [35]. Chemical cleaning commonly is used in conjunction with physical cleaning, such as pressure, vacuum, acoustic energy (ultrasonic bath), and centrifugation. Cleaning grafts to provide additional levels of safety above donor screening can alter their mechanical properties and osteoinductivity [16]. It was reported that cortical bone cleaning, including a hydrogen peroxide step, causes a linear reduction in the osteoinductivity of the bone with increasing exposure time [20]. Hydrogen peroxide is an oxidizing chemical, with the ability to compromise osteoinductivity and bone structural proteins, and thus could affect in vivo graft healing [14, 20]. A loss or reduction of osteoinductivity attributable to processing could cause a decrease or delayed healing response in vivo. In addition, reduction in the strength of the bone structural proteins could result in graft failure, thus negatively affecting graft healing.

Although graft healing in vivo can be affected by processing bone allografts [1, 9, 12, 17], some reports discuss the effects of gamma radiation or ethylene oxide on graft healing [1, 9]. One article discusses the effects of pasteurizing bone grafts and the related effects on healing, where radiographs and histomorphometric analysis were used to examine healing in rabbit ulnas [17].

The ulnar diaphyseal defect in dogs is an established model for examining cortical bone graft healing [16, 18, 19, 30, 31, 34, 36, 41, 43, 47]. The methods of fixation in this model include no fixation [16, 19, 43], an intramedullary Steinmann pin [30, 41], or a plate [34, 46]. Key reported that a defect size that is 1.5 times the diameter of the ulna leads to nonunion [36]. In this model, it was observed that bone healing and remodeling are better with autografts than with allografts, although variability in healing and remodeling is observed [18, 19, 31, 47].

During aseptic processing, the bone is débrided, cut to specification, and cleaned. Cleaning using a standard procedure includes a nonionic detergent soak in an ultrasonic bath followed by a static soak in denatured ethanol [20].

A new cleaning procedure was developed for cortical bone grafts that uses sequential soaks in a nonionic detergent, hydrogen peroxide, and specially denatured alcohol (Recipe SDA 3-C, ethanol denatured with isopropanol)

[14] with all soaks in a temperature-controlled ultrasonic bath at $34^{\circ} \pm 1^{\circ}\text{C}$. The new procedure provides greater than 6 logs reduction in viral clearance for most viruses [20]. The viral clearance properties of the standard procedure have not been characterized; however, from the study of each step of the new procedure we can infer the standard procedure would provide some amount of viral clearance for RNA and DNA enveloped viruses along with nonenveloped RNA picornaviruses (such as hepatitis A virus and human poliovirus type 1), but would have no effect on nonenveloped DNA parvoviruses (such as human parvovirus B19) (Fig. 1). Although, it was reported that the new procedure affects osteoinductivity with increasing exposure time to H_2O_2 , the effect on osteoinductivity is negligible with a 1 hour exposure, and the gain in viral clearance is substantial [7, 20].

Fig. 1 The figure highlights the differences in the new and standard processing techniques. (Reproduced with permission from Springer: DePaula CA, Truncala KG, Gertzman AA, Sunwoo MH, Dunn MG. Effects of hydrogen peroxide cleaning procedures on bone graft osteoinductivity and mechanical properties. *Cell Tissue Bank*. 2005;6:287–298.)

We hypothesized the new processing procedure would not adversely affect graft healing as determined by radiographic, histologic, and histomorphometric evaluations when compared with the standard processing procedure. In addition, we hypothesized autograft healing would be superior by the same parameters when compared with the new and standard processing procedures.

Materials and Methods

We implanted bilateral intercalary allografts and autografts into the right and left ulnas of 13 skeletally mature male coonhounds. Each animal received two allografts, one autograft and one allograft, or two autografts (Table 1). Allografts were processed using the standard procedure or the recently developed new process. A 14th animal was used in this study and served as the initial bone graft donor. Power analysis revealed that to detect a mean difference of one in the radiographic scoring of each graft junction, the sample size needed to obtain a power of at least 0.80 ($\alpha = 0.05$) was 20 junctions. The same sample size was needed to determine a minimum difference of 25% in the histologic scoring. We had 13 canines available for this study, with a total of 26 limbs; thus, there were 26 grafts and 52 graft junctions available for review.

The allografts used in this study were prepared by the Musculoskeletal Transplant Foundation (Edison, NJ) staff using the standard or new technique. After sterile recovery of intercalary ulnar grafts from the left and right forelimbs of the initial donor animal, the bone segments were shipped on dry ice to the Musculoskeletal Transplant Foundation facility for final cleaning and processing. One graft was processed using the standard bone graft processing protocol and the other was processed using the sponsor's new bone

graft processing procedure. After completion of processing, the specimens were shipped on dry ice to the study site in sterile packing and ready for surgical implantation. The initial donor animal was used to acquire grafts for the first animal surgery; subsequent surgeries yielded grafts that then were processed and transplanted according to the study design.

The animals fasted overnight before surgery. Thirty minutes before skin incision, 500 mg cefazolin was given intravenously. On the day of surgery, the animals were sedated, intubated, and anesthetized using a combination of thiopental sodium IV (10 mg/kg) and 1% isoflurane as an inhalation anesthetic. Once surgical anesthesia was attained, both forelimbs were shaved and prepared for surgery following standard protocols for asepsis. All surgeries were completed by an orthopaedic surgeon. After the forelimbs were draped, a 10-cm longitudinal skin incision was made centered over the lateral aspect of the ulna. The fascia and underlying tissues were bluntly dissected down to the periosteum of the ulna. Retractors were inserted, and a middiaphyseal 2.5-cm section of bone was removed from the ulna using an air-powered oscillating saw and constant saline irrigation. A standard allograft, new allograft, or autograft was implanted based on the study design. The grafts were tested for fit and adjusted if deemed necessary. The bone grafts were retained in the defect using a seven-hole, 2.7-mm dynamic compression plate (Synthes, Paoli, PA). The three end holes were located in the proximal and distal ends of the resected ulna, while the central hole was located over the graft. The plate and graft were secured using 2.7-mm cortical bone screws at all locations with the graft fixed with standard interfragmentary compression (Fig. 2). The tissues then were closed in multiple layers using resorbable sutures and final skin closure achieved using nylon sutures. The surgical site then was covered with a sterile dressing. An identical procedure was performed on the contralateral ulna using the autograft from

Table 1. Study design

Dog number	Left side treatment	Right side treatment
1	New	Standard
2	Standard	New
3	Standard	New
4	Standard	New
5	Standard	New
6	New	Standard
7	Standard	New
8	Autograft	New
9	Standard	Autograft
10	Autograft	Autograft
11	Autograft	Standard
12	New	Autograft
13	Autograft	Autograft

Fig. 2 Surgical placement of a graft is shown.

the contralateral side or allograft according to the study design. The section of removed bone was inserted immediately into a sterile container and placed in an insulated container on dry ice if needed for processing or use as an autograft according to the study design. If the study design required transplantation of an autograft, the opposite surgical limb remained surgically exposed, sterilely, until the autograft could be harvested and transplanted from the contralateral limb.

After completion of the procedure, a 75- μ g fentanyl patch was placed and the animals were moved to a special recovery cage where they remained for the first week postsurgery. This cage limits movement of the animal and therefore reduces the risk of perioperative complications. Morphine (0.5 mg/kg) was given IM hourly as needed until 12 hours after fentanyl patch placement. Cefazolin (20 mg/kg) was given twice daily for 3 days postoperatively.

Lateral views of each graft site were taken to confirm graft placement postoperatively and at 90 days to assess displacement of the graft, plate, or screws.

Ninety days after surgery, the animals were euthanized using an intravenous barbiturate overdose in accordance with standard AVMA guidelines. Following euthanasia, the forelimbs were dissected down to reveal the ulna and the graft site. The grafts then were removed by making two cuts in the host's ulna immediately proximal and distal to the attached bone plate. In this manner, the integrity of the host-graft interface was ensured.

The plates were removed after resection. Using a Hewlett-Packard Faxitron Model 43805 N (Hewlett-Packard, Palo Alto, CA) and Kodak Min-R film (Kodak, Rochester, NY), each specimen was subjected to high-resolution radiographic imaging (Fig. 3). These images were used to subjectively evaluate graft integrity and quality of the new bone formed at the graft-host interface. Three blinded observers (JB, RDH, RP) scored the radiographs, using a modified system developed by Stevenson et al. [50] (Table 2). This scoring system rates the distal and proximal unions and appearance and integrity of the host-graft interface, which are combined for a maximum score of eight for each junction. The scores then are added for a maximum score of 16. Interobserver agreement for the radiographic scoring was assessed using Fleiss kappa statistics [57].

The retrieved samples were fixed by placing them in 40% ethanol for at least 72 hours. After fixation, each specimen was embedded in polymethylmethacrylate (PMMA) following a standard protocol for large undecalcified bone specimens. After fixing, the samples were dehydrated for a week in graded alcohols to reach 100% ethanol. Once dehydration was complete, the samples were infiltrated in PMMA I, PMMA II, and PMMA III with increasing amounts of catalyst for 5 to 7 days at each step. The samples then were transferred to a final PMMA

Fig. 3A–B High-resolution (A) anteroposterior and (B) lateral radiographs show a graft processed with the new cleaning process. This graft received a total radiographic score of 14.7/16.

Table 2. Radiographic scoring system [49]

Parameter	Points
Quality of union	
Nonunion	0
Possible union	1
Radiographic union	2
Appearance of graft on anteroposterior view	
Severe resorption	0
Moderate resorption	1
Mild resorption	2
Intact	3
Appearance of graft on lateral view	
Severe resorption	0
Moderate resorption	1
Mild resorption	2
Intact	3
Total points	8

solution and placed in a water bath (37°C) to polymerize. Once polymerized, two midsagittal wafer sections were cut from each block of tissue. The rough-cut wafer section was

mounted on a Plexiglas® slide (Altuglas International, Philadelphia, PA) and ground and polished to a nominal thickness of 75 μm .

Two of the ground and polished slides from each specimen were stained with a combination of Sanderson's Rapid Bone Stain (Surgipath Medical Industries, Richmond, IL) and Van Gieson's picrofuchsin (Fig. 4). When stained using this protocol, mineralized tissue appears orange to red, collagen fibers green to green-blue, osteoid yellow-green, and muscle fibers blue to blue-green. After staining, the slides were digitally photographed and subjected to histologic image analysis.

We (JB, RDH, RP) used a histologic scoring system, modified from Bos et al. [7] and developed for this dog model. For a given ulna specimen, two slides were evaluated by three blinded observers (JB, RDH, RP). The proximal and distal graft-host interfaces were evaluated separately. For the proximal interface, cortical bridging was determined on each of the two slides (two cortices per slide, a total of four cortices). The interface received a percent healing score according to the number of cortices bridged by newly formed woven bone: zero cortices bridged = 0%; one cortex bridged = 25%; two cortices bridged = 50%; three cortices bridged = 75%; and four cortices bridged = 100%. The same scheme was used for the distal interfaces. Thus, the scoring system provided for a maximum score of 100% and a minimum score of 0% in four increments of 25% for each graft interface. When evaluating the proximal and distal junctions separately, a histologic score of zero was considered a nonunion. Interobserver agreement for the histologic scoring was assessed using Fleiss kappa statistics.

A histomorphometric evaluation of the host-graft interface was conducted (Image-Pro® Plus; Media Cybernetics, Inc, Bethesda, MD). The areas analyzed spanned a 1-cm segment, 0.5 cm proximal and 0.5 cm distal to each interface. Thus, for each ulna specimen, the proximal and distal graft-host interfaces were histomorphometrically analyzed separately and then jointly. The metrics analyzed were total bone area in the interfacial region and total endosteal/intramedullary bone in the interface region (Figs. 5, 6).

Fig. 4 This histologic slide shows a graft processed with the new cleaning process. This graft received a 100% histologic union score (Stain, Sanderson's Rapid Bone Stain and Van Gieson's picrofuchsin; original magnification, $\times 7$).

Fig. 5 The total interface area measurement is shown.

Fig. 6 Endosteal bone area measurement is shown.

Differences in the radiographic scores among the three graft types (allografts processed with the new technique, standard-processed allografts, and autografts) were assessed using the Kruskal-Wallis nonparametric test. Likewise, differences in the subjective histologic scores and histomorphometric scores among the three graft types were assessed using the Kruskal-Wallis test. Means and standard deviations were determined in addition to

medians. We used SigmaStat[®] for Windows (SPSS Inc, Chicago, IL) for all analyses.

Results

The new processing procedure did not adversely affect graft healing, as determined by radiographic, histologic, and histomorphometric evaluations, when compared with the standard processing procedure. In addition, we were unable to show a difference in union when autograft was compared with the new and standard processing procedures when subjected to the same evaluation.

Radiographic evaluation revealed no differences in mean radiographic scores for new grafts, standard grafts, and autografts for distal ($p = 0.912$), proximal ($p = 0.963$), or combined interfaces ($p = 0.901$) (Table 3). The overall nonunion rate was four of 52 (7.7%). Two of these nonunions were in grafts treated with the new procedure, one was treated with the standard procedure, and one was an autograft. Three of the nonunions were proximal and one was distal. The interobserver variability of the radiographic scoring was $k = 0.112$.

The histologic analysis showed no differences in mean histologic scores for new grafts, standard grafts, and autografts for distal ($p = 0.118$), proximal ($p = 0.930$), or combined interfaces ($p = 0.917$) (Table 4). The interobserver variability for the three observers was $k = 0.432$.

Table 3. Summary of radiographic scoring

Interface	Radiographic score (mean $\pm$ SD, median)			p Value
	New	Standard	Autograft	
Proximal and distal	13.96 $\pm$ 2.22 14.70	14.71 $\pm$ 0.89 14.70	14.10 $\pm$ 1.76 15.00	0.901
Proximal	6.68 $\pm$ 1.20 7.00	7.01 $\pm$ 0.98 7.00	6.88 $\pm$ 1.10 7.30	0.963
Distal	7.30 $\pm$ 1.52 7.85	7.69 $\pm$ 0.37 7.70	7.23 $\pm$ 1.83 8.00	0.912

Table 4. Summary of histologic scoring

Interface	Histologic score (mean $\pm$ SD, median)			p Value
	New	Standard	Autograft	
Proximal and distal	83.89 $\pm$ 21.19 91.65	88.11 $\pm$ 17.93 96.00	87.13 $\pm$ 20.62 95.95	0.917
Proximal	73.22 $\pm$ 42.87 100.00	79.67 $\pm$ 34.30 100.00	86.50 $\pm$ 19.32 91.70	0.930
Distal	94.44 $\pm$ 7.32 100.00	96.33 $\pm$ 8.43 100.00	88.89 $\pm$ 33.33 100.00	0.118

Table 5. Summary of histomorphometric analyses (endosteal bone area)

Interface	Endosteal bone area in mm ² (mean $\pm$ SD, median)			p Value
	New	Standard	Autograft	
Proximal and distal	18.02 $\pm$ 7.21 15.70	18.69 $\pm$ 6.70 15.70	18.92 $\pm$ 5.43 17.14	0.777
Proximal	11.45 $\pm$ 3.05 12.33	12.03 $\pm$ 4.63 12.25	13.50 $\pm$ 5.08 12.91	0.766
Distal	6.57 $\pm$ 5.81 4.74	6.66 $\pm$ 4.56 5.50	5.42 $\pm$ 3.74 4.93	0.969

Table 6. Summary of histomorphometric analysis (total bone area)

Interface	Total bone area in mm ² (mean $\pm$ SD, median)			p Value
	Improved	Standard	Autograft	
Proximal and distal	83.51 $\pm$ 24.21 77.43	83.31 $\pm$ 16.49 75.03	85.32 $\pm$ 10.43 83.63	0.899
Proximal	46.06 $\pm$ 12.39 44.85	44.64 $\pm$ 9.81 42.33	44.95 $\pm$ 8.08 47.87	0.977
Distal	37.45 $\pm$ 12.54 30.67	38.67 $\pm$ 8.14 35.11	40.37 $\pm$ 7.55 38.80	0.573

Histomorphometric analysis revealed no differences in mean total areas for new grafts, standard grafts, and autografts for distal ($p = 0.573$), proximal ($p = 0.977$), or combined interfaces ($p = 0.899$). There were no differences in mean endosteal areas for new grafts, standard grafts, and autografts for distal ($p = 0.969$), proximal ($p = 0.766$), or combined interfaces ($p = 0.777$) (Table 5). In addition, there was no difference in total bone area in the proximal ($p = 0.977$), distal ($p = 0.573$), and combined interface ($p = 0.899$) (Table 6).

All animals tolerated the surgical bilateral grafting procedure without incident and to term. On retrieval of the specimens, all were judged to have healed wounds and were free from any signs of infection. There was no indication of any adverse tissue response in any case, and there was no discernable difference in the gross appearance of the tissues between the left and right side implant sites on explantation. Position of the graft, plate, and screws were maintained in all animals.

Discussion

When altering the processing techniques of allografts to improve the safety of transplantation, the effects on healing must be measured. Ideally, the addition of H₂O₂ in

processing to improve the safety of allografts should not adversely affect graft healing. Although, the data suggest the new procedure affects osteoinductivity with increasing exposure time to H_2O_2 , the effect on osteoinductivity is negligible with a 1-hour exposure, and the gain in viral clearance is substantial [8, 20]. Our hypothesis was that the new processing procedure would not adversely affect graft healing, as determined by radiographic, histologic, and histomorphometric evaluations, when compared with the standard processing procedure. In addition, we hypothesized that autograft healing would be superior when compared with the new and standard processing procedures when subjected to the same evaluation.

Although we were unable to detect a difference in union, there are some limitations to the study. First, the number of experimental animals was small at 13, however, as bilateral surgery was performed and each limb assessed at two junctions, the number of junctions assessed was 52, increasing the power of the study despite the limited number of canines. Second, union of allografts and autografts in this study at 90 days was similar and, although autografts are reportedly superior in terms of healing [48], this is not completely unexpected. As animals are bred, the immunologic reaction to allografts used in this study may be minimal. Others have reported that in closely matched animals, allografts and autografts have incorporated [7]. In addition, there was substantial interobserver variability regarding the scoring of radiographs and histologic slides. Finally, the type of fixation, use of interfragmentary compression across the transplant, allowance of weightbearing, and the end point of 90 days are variables that may have affected our overall assessment of union [4, 6]. However, nonunions were detected in all experimental groups, suggesting that despite these variables, detectable nonunions do occur. With an overall nonunion rate of 7.7% (four of 52 junctions), the model produced a nonunion rate similar to that in a human clinical report [42]. Furthermore, the model shows a trend toward nonunions at the proximal ends of the graft-host interface, which is more diaphyseal in location, more often than at the distal end of the graft-host interface, which is more metaphyseal in location, a phenomenon that is mirrored in humans.

Other investigators have confirmed that resection of a length of the ulna equal to twice the diameter in the midshaft leaves a defect which fails to unite [43]. In that study, defects filled with autogenic cortical bone chips showed union at 12 weeks [43]. Others have reported a canine ulna model is useful in detecting differences in union of irradiated and nonirradiated allografts at 150 days [22]. In a similar study of canine ulna intercalary defects, cancellous allogeneic bone block that had been immersed in liquid nitrogen for 3 weeks failed to achieve union at 24 weeks. However, in that study the

periosteum was not retained. Perhaps retention of the periosteum and avoidance of liquid nitrogen in our study contributed to healing at 12 weeks [47].

We found no differences in union in a canine model at 90 days when the new cleaning procedure was compared with the standard procedure and with autograft controls.

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