stryker

Pro-Dense®
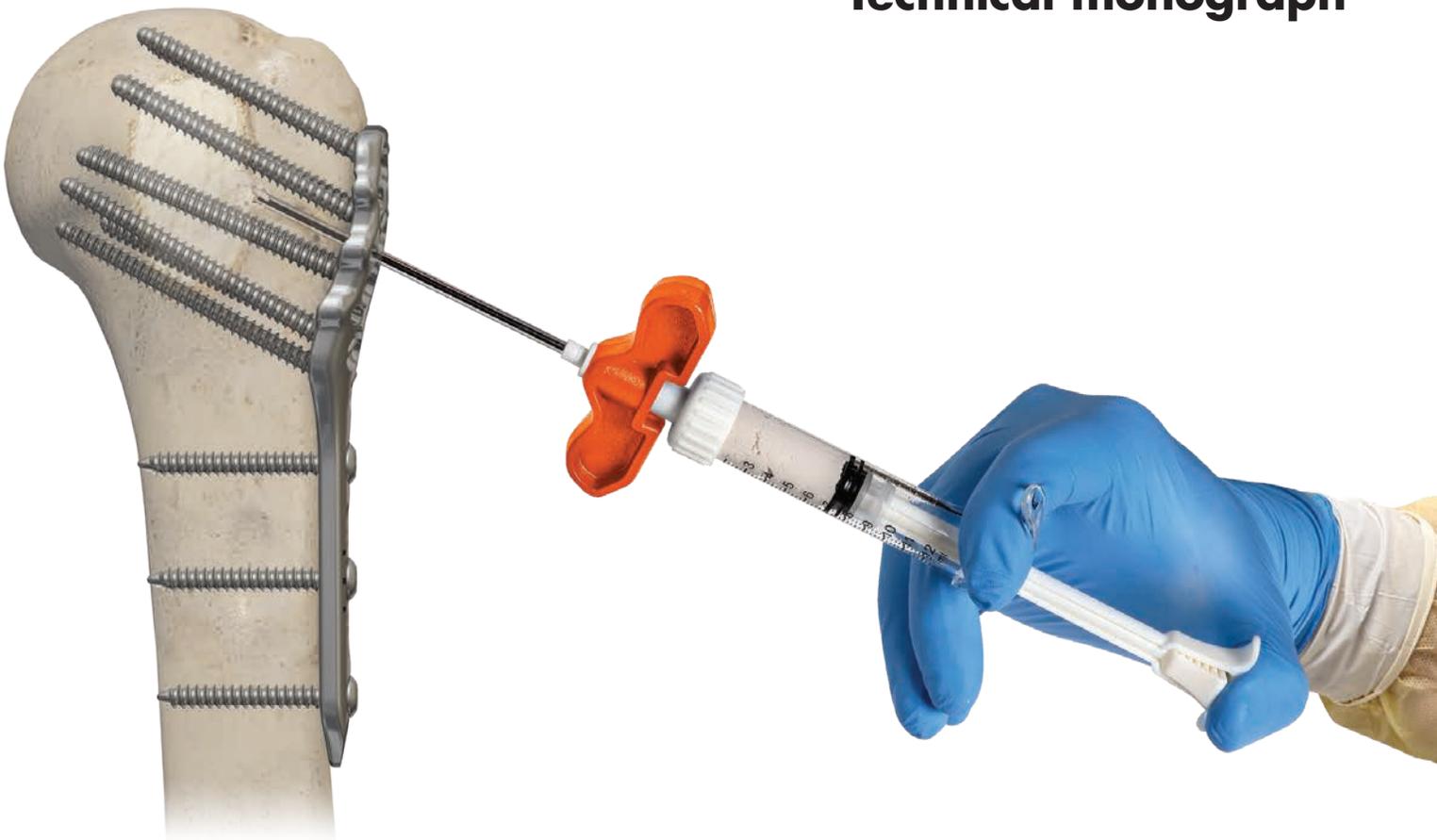## Injectable Regenerative Graft

Technical monograph

In vitro and in vivo observations, and a proposed mechanism of action

Abstract

Pro-Dense Injectable Regenerative Graft is a triphasic calcium, salt-based material that has demonstrated rapid, robust formation of dense new bone in preclinical studies and in clinical results. A proposed mechanism of action for Pro-Dense Injectable Regenerative Graft is discussed. The progression of bone formation involves angiogenesis, protein binding and release, cell differentiation and proliferation, new bone formation, and bone remodeling. Preclinical studies have demonstrated that the calcium sulfate component of the Pro-Dense Graft composite resorbs first, allowing for vascular infiltration into the calcium phosphate scaffold. In vitro studies of VEGF and BMP-2 have shown increased binding and release of proteins from Pro-Dense Graft compared to calcium sulfate. The activity of VEGF released from Pro-Dense Graft has been confirmed in an in vitro study of endothelial cells exposed to released VEGF. Results from ongoing studies have provided the framework for the proposed mechanism of action for Pro-Dense Injectable Regenerative Graft.
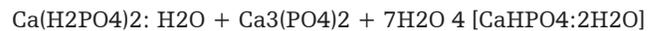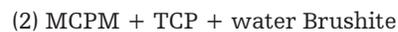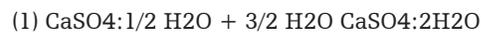
Background

Pro-Dense Bone Graft substitute is a fully synthetic composite material that is designed to be mixed intraoperatively and injected into a bony void to cure in vivo to form a strong ceramic. Results with Pro-Dense Graft in three in vivo preclinical studies have consistently demonstrated predictable, robust bone formation. In a canine metaphyseal defect model, the majority of the graft was resorbed and replaced by new bone over a period of approximately three months. A review of clinical cases indicates that the graft resorbs at a slower rate in humans.

The ability of Pro-Dense Graft to be replaced predictably with robust bone is likely attributable to the unique dissolution and protein-binding properties of the carefully engineered composite. The calcium sulfate/calcium phosphate matrix with TCP granules provides a composite containing 75% calcium sulfate and 25% calcium phosphate (brushite and granular TCP). This paper describes some in vitro and in vivo observations that may help elucidate the mechanism of the tremendous bone formation seen with Pro-Dense Injectable Regenerative Graft.

In situ reaction

The chemistry of Pro-Dense Graft is complex and involves two distinct reactions that occur simultaneously to produce a homogeneous combination of calcium sulfate dihydrate and calcium phosphate surrounding unreacted tricalcium phosphate (TCP) granules. The first reaction is a result of the combination of calcium sulfate hemi-hydrate (CSH) with water to form calcium sulfate dihydrate (CSD). (Equation 1)

Pure calcium sulfate has been used successfully in bone grafting for over 100 years.[1,2] It has long been recognized, however, that in certain applications a somewhat slower resorbing material is desirable. In order to achieve this goal, in Pro-Dense Graft the CSH has been augmented with two calcium phosphate compounds that entail the second, yet simultaneous reaction, to form a mineral known as "brushite." (Equation 2)

(1) $CaSO_4{:}1/2\ H_2O + 3/2\ H_2O\ CaSO_4{:}2H_2O$

(2) MCPM + TCP + water Brushite

$Ca(H_2PO_4)_2{:}\ H_2O + Ca_3(PO_4)_2 + 7H_2O\ 4\ [CaHPO_4{:}2H_2O]$

Because these reactions take place concurrently in the setting graft, the two products are intertwined on the microscopic level and are not distinguishable from one another by either light microscopy or scanning electron microscopy (SEM).

As mentioned previously, in addition to the calcium sulfate and brushite, dense granules of beta-tricalcium phosphate (ß-TCP) have been incorporated into Pro-Dense Graft. These granules are of a sufficient size and density that they are not consumed in the reaction that forms brushite. Therefore, the set graft consists of a distributed aggregate of TCP granules embedded in a matrix of intimately intertwined CSD and brushite crystallites. This is illustrated in Figure 1, which shows a polished cross-section through the middle of a 4.8mm diameter pellet cast from Pro-Dense Graft. The TCP granules are bright white, embedded in the light grey matrix.
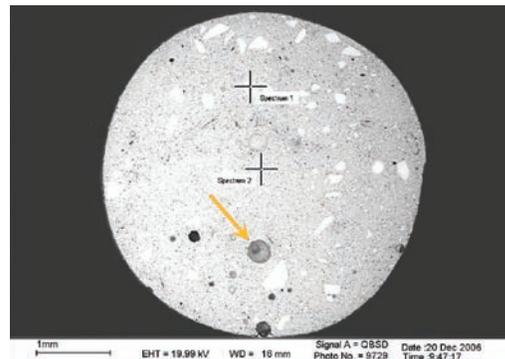

Figure 1: Backscattered electron image of a polished cross-section through a Pro-Dense Pellet. The bright white particles are TCP. Several pore artifacts resulting from the sample preparation are evident in this cross-section (arrow).

Mechanism and kinetics of Pro-Dense Graft resorption

The kinetics of dissolution of Pro-Dense Graft have been studied in an accelerated test in which the pellets are immersed in distilled water at 37°C. Pre-cast and weighed pellets measuring 4.8mm diameter by 3.2mm tall were placed in fritted glass thimbles to allow removal of pellets at test intervals without damaging the pellets. The distilled water was changed daily and the pellets (N=5) were dried and weighed to determine the percent mass remaining. Estimates based on a comparison of the percent pellet remaining in the dissolution test and radiographs from the preclinical animal model show that the in vitro dissolution rates are approximately six times faster than what is seen in vivo.

For this study, pellets of pure calcium sulfate (CSD) were tested in the same fashion as a point of reference for a faster-resorbing material.

At selected times, pellets of Pro-Dense Graft were removed from solution, dried, stabilized with ethyl cyanoacrylate, and embedded in PMMA. Ground and polished ross-sections were subjected to microscopic analysis using backscattered SEM and EDXS (energy-dispersive X-ray spectroscopy), to determine the elemental composition of each material region

Accelerated dissolution results

The comparative dissolution profiles of Pro-Dense Graft and pure CSD are shown in Figure 2. The rate of dissolution as determined by the slope of the curves over the first four days of this accelerated test shows that the design goal of a 50% reduction in weight loss per unit time compared to pure CSD was achieved. Over time, dissolution of the calcium sulfate material in the Pro-Dense Graft exposed a porous matrix of brushite that contained embedded TCP granules. (Figure 3)
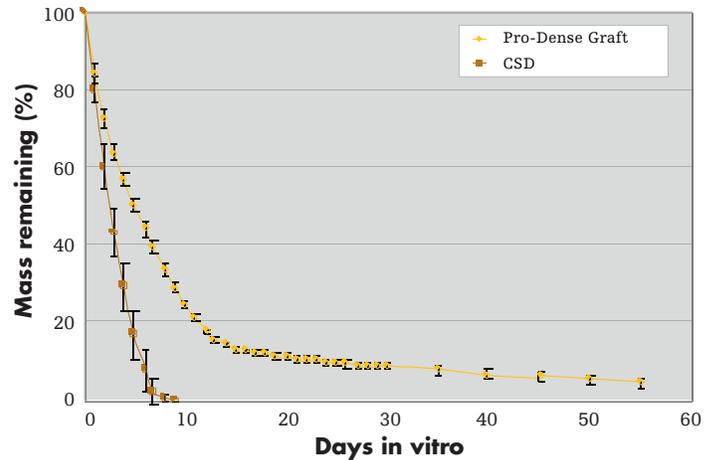

Figure 2: Accelerated in vitro dissolution of calcium sulfate and calcium sulfate/calcium phosphate composite
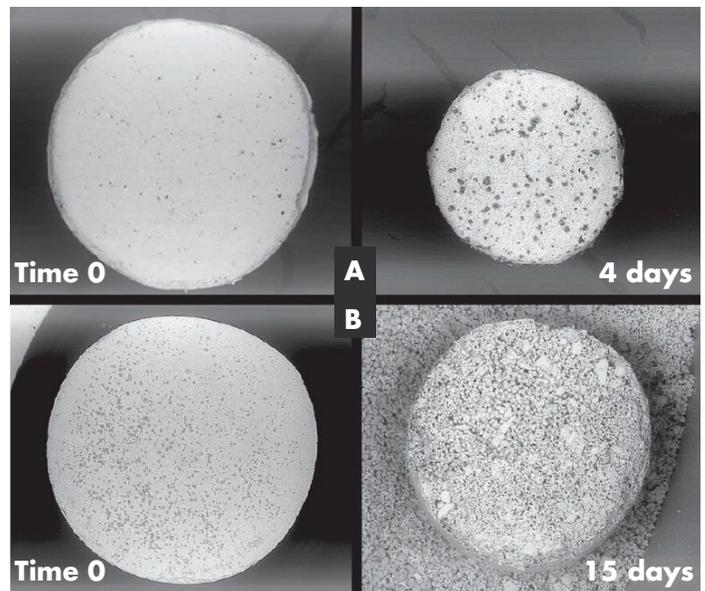

Figure 3: In vitro dissolution of (a) calcium sulfate dihydrate pellets by four days, and of (b) Pro-Dense Graft at 15 days (original magnification 35X). The overall size of the CSD pellet is reduced even at an early timepoint. Much of the size of the Pro-Dense Graft is maintained, even at 15 days, but the calcium sulfate has dissolved to reveal the porous calcium phosphate structure containing TCP granules.

This difference in dissolution rate is thought to be due to the microscopic scale in which the brushite and CSD phases of Pro-Dense Graft are distributed in the matrix of the set graft. As CSD is dissolved from the surface of the pellet, the finely dispersed brushite crystals that are left behind act to slow the diffusion of CSD dissolution products. The resulting local increase in concentration of Ca (calcium) and SO4 (sulfate) ions slows the dissolution rate from the remaining bulk matrix. The physical progression of this process is illustrated in Figures 4-6, which show SEM images of cross sections through embedded Pro-Dense Graft after four, eight, and 12 days of accelerated in vitro dissolution, which has been estimated to occur about six times faster than in vivo.

In these images, intact matrix shows as a light grey and TCP granules as white. The brushite that is evident in the partially dissolved areas shows up as a darker grey material dispersed through the embedding media that has infiltrated into the space previously occupied by the CSD. Elemental analysis of the various regions within the cross-sections confirms the identification. Regions of intact matrix have high values for sulfur concentration while zones that are depleted of CSD show significantly lower sulfur concentrations.

The combination of mass loss data and microscopic analysis leads to the following conclusions regarding the physical-kinetic process of dissolution for Pro-Dense Graft.

1. There is an initial burst of CSD dissolution from the surface of the pellet, which exposes an outer layer of fine brushite crystals and larger TCP granules.

2. The brushite forms a diffusion barrier that slows the rate of CSD dissolution.

3. As dissolution proceeds, brushite crystals on the exterior of the pellet (those that were first exposed) appear to become less dense, indicating that the brushite is also dissolving.

4. The relatively dense region of brushite that surrounds the intact portion of the pellet moves inward as dissolution continues.

5. The TCP granules form a distributed scaffold even after the majority of the CSD and brushite have dissolved. It is likely that some of the brushite remains attached to the TCP and acts to hold the granules in place.
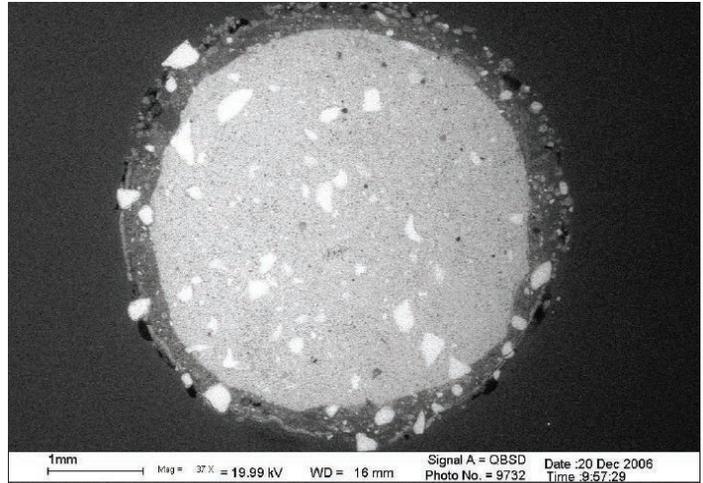

Figure 4: Cross-section through a Pro-Dense Pellet after four days of accelerated in vitro dissolution (which represents approximately 24 days in vivo). The darker region on the outer edge of the pellet represents the area in which calcium sulfate has mostly dissolved, leaving brushite and TCP particles (bright white).
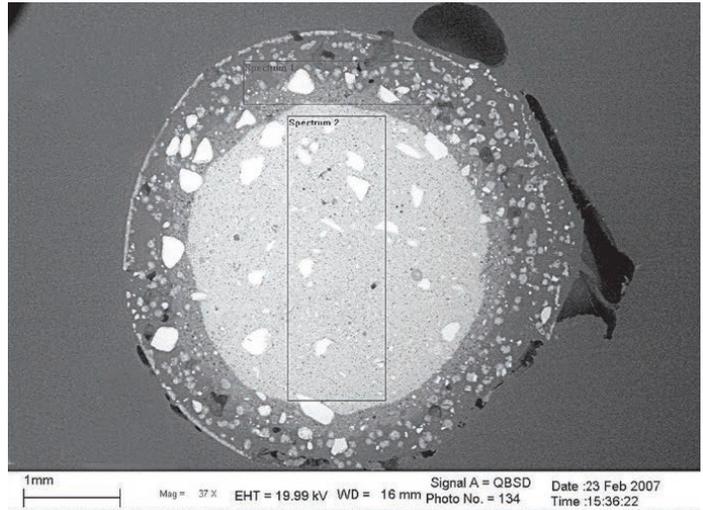

Figure 5: Cross-section through a Pro-Dense Pellet after eight days of accelerated in vitro dissolution (approximately 48 days in vivo). Dissolution of the calcium sulfate continues to progress inward.
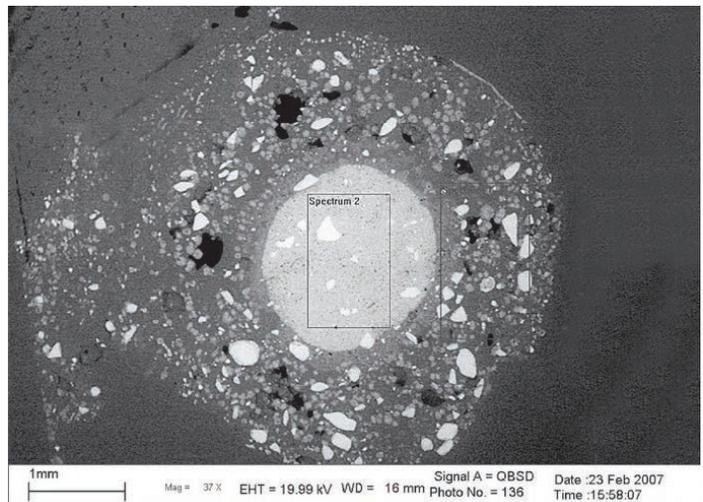

Figure 6: Cross-section through a Pro-Dense Pellet after 12 days of accelerated in vitro dissolution(approximately 72 days in vivo). The region of dissolving brushite is increasing with a subsequent decrease in the region with intact calcium sulfate.

Proposed mechanism of action of new bone formation

In defects treated with Pro-Dense Graft, triphasic resorption facilitates a maximum healing response. This effect, which has also been seen clinically, has been observed and described in a critically sized metaphyseal defect model in the proximal humerus of dogs.[3] Similar results have also been seen in a canine humerus transcortical non-critically sized defect model. Potential contributing mechanisms have also been examined and confirmed in vitro.

Canine defect studies

Pro-Dense Graft has been investigated in three canine studies. The first study involved the creation of non-critical size transcortical defects, (9mm diameter x 20mm long) in the proximal humerus of three mongrel dogs (data on file). Animals were sacrificed at 6 weeks and the implant sites harvested and prepared for histological examination. In this initial study, Pro-Dense Implants were associated with progressive, robust bone growth over time as determined by radiographs. Bone formation was denser than surrounding bone at 6 weeks as observed in histological sections. In the second and third studies, critically sized defects were created in the proximal humerus of mongrel dogs. The cylindrical, longitudinal defects were created by drilling a 13mm diameter x 50mm long hole adjacent to the greater trochanter.[3]

In the second study, one defect was filled with Pro-Dense Graft in each of 10 dogs. At 13 and 26 weeks, the animals were sacrificed and the defect sites harvested for contact radiographs, histology, and mechanical testing. In the third study, six dogs were implanted. Each canine was sacrificed at an early timepoint (2, 3, 4, 6, 8, or 10 weeks) and contact radiographs and histological specimens were prepared. The results were compared to data for autograft and normal bone collected in similar but unrelated studies.

The radiographs and histology from both of these studies clearly illustrated an efficient and constant progression of material resorption and bone growth over time. The Pro-Dense Graft was also associated with the growth of dense new bone in early timepoints and a significant increase in compressive strength of the healed bone compared to normal bone at the earliest timepoint that mechanical testing was employed, 13 weeks.

Representative contact radiographs taken at each timepoint are shown in Figure 7. As early as the first timepoint, 2 weeks, the material showed resorption and the remaining bolus was surrounded by a thin region devoid of calcified components, which was immediately adjacent to a region of particularly dense new bone. By 8 weeks, less than 50% of the material remained and the front of new bone had continued to move inward. By 13 weeks, the bolus of material had almost completely resorbed and was replaced with new bone. By 26 weeks, the new bone in the defect had remodeled to resemble normal bone.
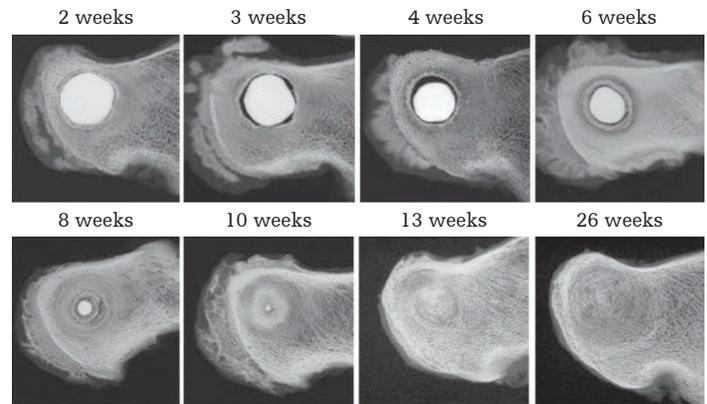

Figure 7: Representative contact radiograph images showing the degradation of Pro-Dense Graft and infill of new bone over time.

Examination of the histology images and histomorphometric analysis at each timepoint as well as compressive strength measurements taken at 13 and 26 weeks support the progression seen in the contact radiographs. Histomorphometry data of Pro-Dense Implants were compared to data from autograft implants collected in unrelated studies.

Prior to 8 weeks, the percent area of new bone in the Pro-Dense Graft-treated sites was lower than in autograft-treated sites or normal bone due to the presence of a significant amount of residual graft. By 8 weeks, however, a majority of the graft had resorbed, and the percentage of new bone surpassed that of the autograft and allograft groups. At 13 and 26 weeks, the amount of new bone in Pro-Dense Graft-filled sites compared with autograft-treated and allograft-treated sites showed a similar profile as mechanical strength, indicative of remodeling to normal bone.[3] (Figure 8) At 13 weeks, the compressive strength of new bone in sites receiving Pro-Dense Graft was almost six times (5.7X) that of sites receiving autograft (in an unrelated study), well over 10 times (17X) vs. allograft CBM, and greater than three times (3.7X) that of normal bone taken from a similar anatomical site. As most of the implant had resorbed by 13 weeks (>90%), the increase in strength can primarily be attributed to increased strength of the healing bone as further confirmed by the dense bone and minimal residual graft seen in histological specimens. By 26 weeks, new bone had remodeled and exhibited mechanical properties only slightly higher than those of normal bone.
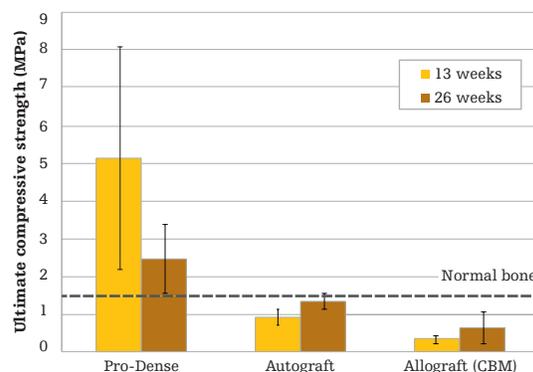

Figure 8: Compressive strength of new bone in defect sites treated with Pro-Dense Graft. Data for autograft-treated sites, allograft-treated sites, and normal bone were collected in similar, unrelated studies.

Progression of new bone formation

The formation of new bone is described as a progression of five steps, which repeat as the graft is resorbed until the entire graft site is filled with new, remodeled bone.

1. Angiogenesis

As with all healing, angiogenesis is a key early event in the repair of injured bone. Immediately following an injury, growth factors and other proteins flood the region to attract and stimulate cells to initiate the healing process, including enabling the formation of new blood vessels. As demonstrated in vitro, the calcium sulfate component is the first phase of the Pro-Dense Implant material to be resorbed. As the calcium sulfate is resorbed in vivo by one or more mechanisms including dissolution and osteoclastic resorption (Figure 9),[4] the calcium phosphate (i.e., brushite and TCP) scaffold with interconnected pores is revealed, allowing for infiltration into the implant by newly formed blood vessels. (Figure 10)
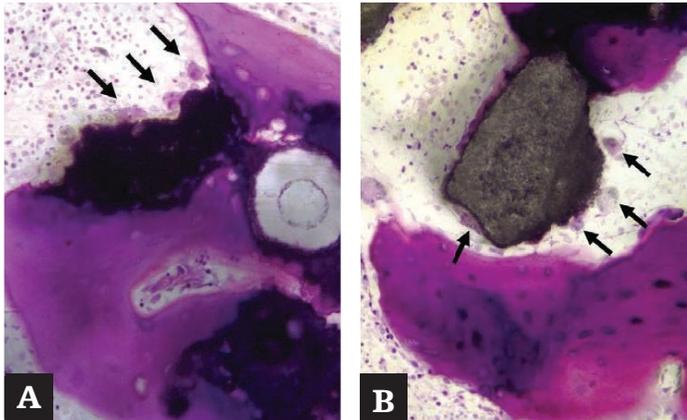
Figure 9: Osteoclasts (arrows) resorbing (a) calcium sulfate/ brushite material, and (b) ß-TCP granules, in a canine proximal humerus defect site treated with Pro-Dense Graft, 13 weeks post-implantation. (Basic fuschin and toluidine blue, 300X)
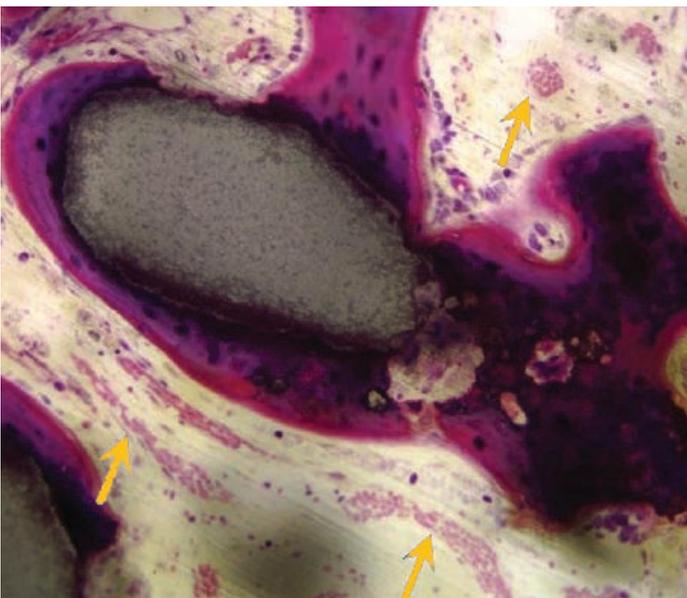
Figure 10: New blood vessels (arrows) adjacent to Pro-Dense Graft in a canine proximal humerus defect site implanted with Pro-Dense Graft, 6 weeks post-implantation. (Basic fuschin and toluidine blue, 300X)

2. Protein binding and release

As new blood vessels infiltrate the defect region and the calcium phosphate scaffold, cytokines and growth factors are transported into the implant/defect interface region. Angiogenic factors such as VEGF (vascular endothelial growth factor) and FGF (fibroblast growth factor) play a key role in the development of new blood vessels, including the chemoattraction and induction of proliferation of endothelial cells.[5,6]

An in vitro study of Pro-Dense Graft specimens exposed to radiolabelled VEGF or radiolabelled BMP-2 (bone morphogenetic protein-2) has shown that the proteins preferentially bind to Pro-Dense Graft compared to pure calcium sulfate.[7] Briefly, Pro-Dense Graft and calcium sulfate pellets were incubated in PBS with 20 μg/mL VEGF or BMP-2 at 37°C for 1 hour (4 replicates for VEGF; 6 replicates for BMP-2). After rinsing to remove loosely adhered proteins, pellets were placed in tubes and the radioactivity, indicative of adhered proteins, was measured with a gamma counter. There was a trend toward higher adsorption of VEGF on Pro-Dense Graft although the difference was not significant. (Figure 11) Significantly greater binding of BMP-2 was measured from Pro-Dense Graft compared to CSD (p=0.026). (Figure 11)
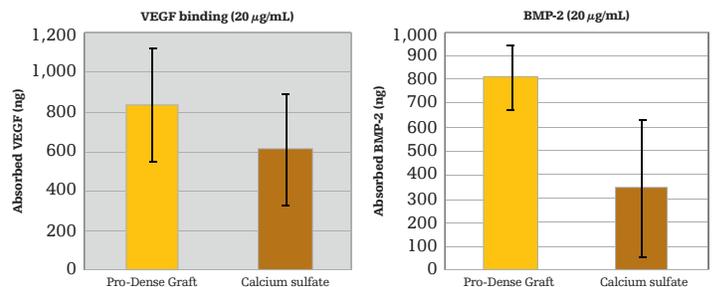
Figure 11: Proteins adhered to pellets soaked in 20 μg/mL VEGF or BMP-2. Bars represent standard deviations.

3. Cell proliferation and differentiation

Following injury and implantation of biocompatible materials, active proteins such as TGF-ß (transforming growth factor beta) attract regenerative cells to the defect/implant interface region.[7] Released proteins and other proteins in the surrounding area also incite cell proliferation and induce differentiation of stem cells to osteoblast progenitors and then to osteoblasts. Factors which enhance proliferation of osteoblastic stem cells, osteoblast precursors, and/or osteoblasts include TGF- ß, PDGF (platelet derived growth factor), and IGF-I and IGF-II (insulin-like growth factor-I and -II).[8-13] Bone morphogenetic proteins (BMPs), also referred to as osteoinductive factors, play a critical role in the differentiation of stem cells to preosteoblastic and osteoblastic cells.[14,15,16]

In vitro results have indicated that proteins released from the Pro-Dense Scaffold are active and stimulatory.[7] In the demonstrative study, Pro-Dense Disks were soaked in a 20 μg/mL VEGF solution for 1 hour and then rinsed to remove loosely adhered proteins. Disks were then incubated in PBS solution and eluates were drawn at 24, 48, and 72 hours, with replenishment of the PBS solution after each eluate aspiration. Human umbilical vascular endothelial cells (HUVEC) were seeded onto transwell inserts (pore size 8 μm), which were placed in media dosed with the eluates for 4 hours. Following incubation, cells that had migrated from the inserts to the bottom of the wells were trypsinized and counted.

There was a trend toward an increase in cellular migration in wells treated with Pro-Dense/VEGF eluates vs. control eluates (i.e., Pro-Dense Graft/PBS alone) (Figure 12), indicating that the released protein was active and stimulatory. Additionally, the number of migrated cells was relatively consistent at the three timepoints (24, 48, 72 hours), supporting a pattern of sustained release of the protein from the Pro-Dense Graft specimens.
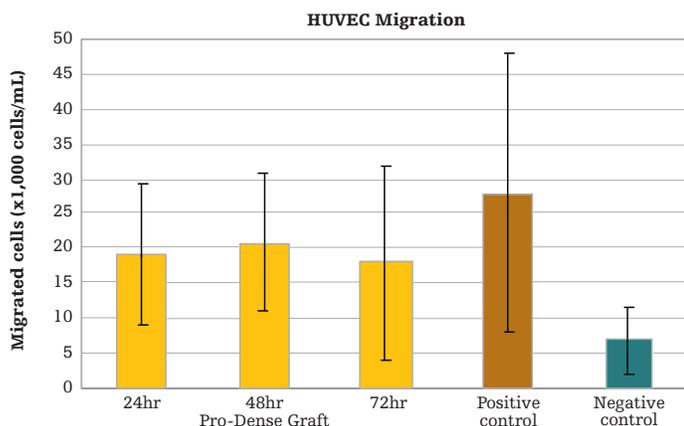
HUVEC Migration

Figure 12: HUVEC cells migrated over time in response to VEGF absorbed and then eluted from Pro-Dense Graft samples. The positive and negative controls were PBS with 40 ng/mL VEGF and PBS alone, respectively. Bars represent standard deviations.

4. New bone formation

In the canine models discussed, new bone formed directly adjacent to Pro-Dense Graft. Osteoblasts recruited to the area formed the new bone via tramembranous ossification. Although new bone growth in long bones is typically formed via endochondral ossification (i.e., the inclusion of an intermediary cartilage scaffold), osteoblasts adjacent to Pro-Dense Graft directly laid down osteoid tissue, which was then mineralized to form new bone. (Figure 13) Osteoblasts continued to regenerate new bone, eventually surrounding residual Pro-Dense Graft materials. (Figure 14) Over time, the remainder of the Pro-Dense Graft was resorbed, again due in part to osteoclastic resorption.

This progression of new bone formation is consistent with the theory presented regarding resorption shown by in vitro dissolution studies. As seen histologically, new bone formation starts at the edge of the defect, presumably following new blood vessels into the porous brushite/TCP matrix. As resorption of the calcium sulfate and calcium phosphate progresses inward toward the center of the original Pro-Dense Graft bolus, new bone growth follows until the majority of the implant material is gone.
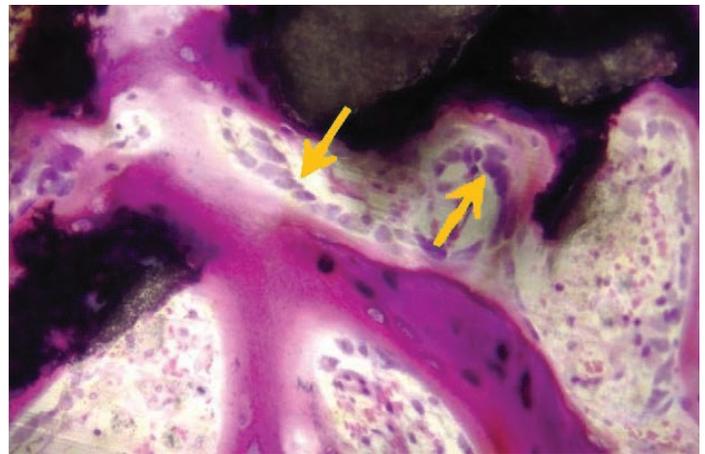
Figure 13: Osteoblasts (arrows) adjacent to the osteoid (very light pink) and calcified new bone (darker pink) laid upon residual Pro-Dense Graft material, in a canine transcortical defect at 6 weeks. (Basic fuschin and toluidine blue, 300X)
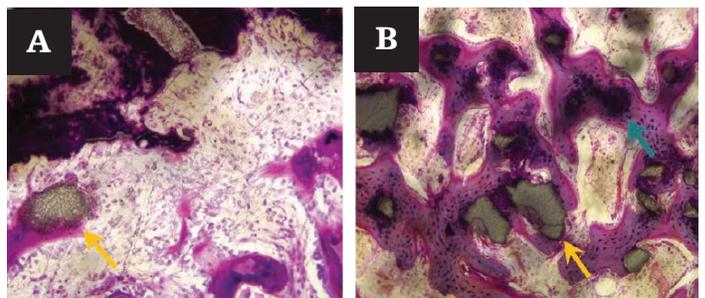
Figure 14: Newly formed bone (dark pink) adjacent to residual Pro-Dense Graft material at (A) 2 weeks (154X), and (B) at 6 weeks (75X) in a proximal humerus defect and transcortical defect, respectively, in dogs. TCP granules are light grey (yellow arrow) and brushite or calcium sulfate are dark material. (green arrow). (Basic fuschin and toluidine blue)

5. Bone remodeling

Following formation of newly woven bone, remodeling occurs in response to mechanical loading (Wolff's Law). Over time, new bone in the defect site remodels to resemble normal bone. (Figure 15)
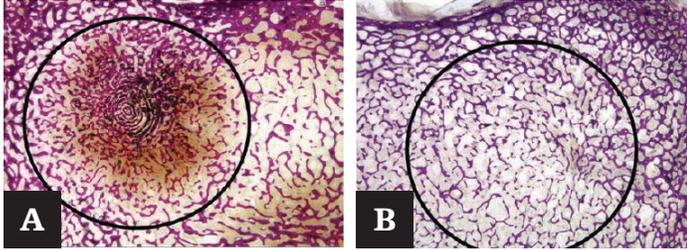

Figure 15: Newly formed bone in the proximal humerus of dogs at: (A) 13 weeks, and (B) 26 weeks. The circles approximate the original defects. At 13 weeks, there is still some residual implant material, and the bone formation around the implant material is particularly dense. By 26 weeks, the material is mostly resorbed and the bone is continuing to remodel toward normal bone architecture. (Basic fuschin and toluidine blue, 10X)

Clinical observations

Pro-Dense Injectable Regenerative Graft has been in clinical use globally for over 12 years now in a variety of clinical applications, including benign bone cysts/tumors, osteolytic lesions, trauma, core decompressions for AVN, autograft harvest and hardware removal backfill sites among other applications. Pro-Dense Graft has demonstrated consistent material resorption, accompanied by predicable, robust new bone formation.[17-27] The region of protein absorption, protein release, and new bone formation surrounds the implant material. Computed tomography (CT) scans have typically shown a thin, translucent layer, which is believed to represent the layer of new, unmineralized osteoid. This thin region advances inward as graft resorption and healing progress. CT scans also show a layer of denser bone adjacent to the healing defect site at early timepoints—which decreased over time—that is consistent with radiographic results of healing defects in the canine studies.[3]

Conclusions

Ongoing pre-clinical in vivo and in vitro studies offer insight into the possible mechanism of action that makes Pro-Dense Injectable Regenerative Graft a powerful graft for healing bone defects. Studies thus far have shown that the graft provides unique dissolution properties and the ability to bind and release proteins at the implant/defect interface. This provides an ideal environment for the direct deposition of bone, resulting in a slow-resorbing matrix that propagates healing across the defect. This may explain the predictable, robust bone formation seen in the animal models and in clinical experience.

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