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BMP-2 impregnated biomimetic scaffolds successfully induce bone healing in a marginal mandibular defect

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Abstract

Educational Objective: To investigate the ability of an osteoconductive scaffold to heal a clinically common mandibular defect with BMP-2 in an animal model.

Objectives: To test the osteoregenerative potential and dosing of bone morphogenetic protein-2 (BMP-2) impregnated biomimetic scaffolds in a rat model of a mandibular defect.

Study Design: Prospective study using an animal model.

Methods: Varied doses of BMP-2 (0.5, 1, 0.5 and 0.5 in microspheres, 5, 15 µg) were absorbed onto a biomimetic scaffold. Scaffolds were then implanted into marginal mandibular defects in rats. Blank scaffolds and unfilled defects were used as negative controls. Two months postoperatively, bone healing was analyzed with micro-computerized tomography (microCT).

Results: MicroCT analysis demonstrated all doses of BMP-2 induced successful healing of marginal mandibular defects in a rat mandible. Increasing doses of BMP-2 on the scaffolds produced increased tissue healing with 15 µg demonstrating significantly more healing than all other dosing ($p < 0.01$).

Conclusions: BMP-2 impregnated biomimetic scaffolds successfully induce bone healing in a marginal mandibular defect in the rat. Percentage healing of defect, percentage of bone within

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healed tissue and total bone volume are all a function of BMP-2 dosing. There appears to be an optimal dose of 5 µg beyond which there is no increase in bone volume.

Keywords

Biomaterials; Osteogenesis; Craniofacial; Mandible; Tissue Engineering

Introduction

Craniofacial defects are a diverse and challenging clinical problem. Defects in the facial skeleton are currently treated with autologous tissue in the form of bone grafts or flaps (Canady); however, transfer of autologous bone is hindered by prolonged operative time, limited tissue availability and donor site morbidity (Zermatten). Currently, the preferred methods of reconstruction of segmental mandibular defects are bony vascularized flaps (Blackwell). Although free flaps are a reliable means of reconstruction, they require lengthy surgeries that carry a 20.5% (Suh) risk of perioperative medical complications (Blackwell, Urken). An alternative means of reconstruction has the potential to spare patients life-threatening medical morbidity associated with the current standard of care.

The use of growth factors, including bone morphogenetic proteins (BMPs), transforming growth factor, fibroblast growth factor and insulin-like growth factor, has been investigated as an alternative to autologous grafting. BMPs, have been shown to stimulate bone regeneration (Cheng 2003; Cowan 2007), and BMP-2 is currently approved for use in spinal fusions, long bone fractures, maxillary sinus augmentation, and localized alveolar ridge defects. The concentrations of endogenous BMPs are several orders of magnitude lower (nanograms compared to milligrams) than the doses required for clinical use (Valentin). Supraphysiological doses required for clinical use, may lead to unintended consequences such as heterotopic bone formation and pleiotropic non-bone-specific effects (Valentin).

In part the dosing differential is due to the acute delivery of exogenous BMP, which has a half-life of hours (Giannobile); however, through the use of scaffolds BMP can be delivered to bony wounds at more physiologic levels (Lee 1994). Ideally, growth factor delivery systems must be biocompatible, biodegradable, made sterile for implantation, and act as a structural support for cell colonization. Poly(lactic-co-glycolic acid) (PLGA) has been studied as a scaffold for bone regeneration both *in vitro* and *in vivo* (Athanasίου, Kontakis). PLGA scaffolds can be precisely manipulated through three-dimensional printing (Lee 2005) to mirror a craniofacial defect with tremendous accuracy. They are capable of accommodating cell migration through tightly controlled porosity (Lee 2005), and may be loaded with various growth factors for both experimental and clinical applications. (Lee 2005). Importantly, PLGA scaffolds allow for manipulation of the kinetics of growth factor release through encapsulation of the growth factors in PLGA microspheres (Peng 2008). As such, the exact spatial and temporal delivery of growth factors can be tailored based on the desired effect.

Rat animal models of partial mandibular defects have been used to investigate the osteogenic potential of BMP-2 but these studies have been done in defects that do not represent large marginal mandibular defects often created in tumor ablation surgery

(Schielaphke, Zellin), or with scaffolds that lack structural integrity to be shaped into three-dimensional structures (Issa) or in fact induce bone growth (Arosarena 2005). We have recently described a novel animal model for the study of composite mandibular resection in the rat (Sidell), and the present study employs a marginal mandibular model.

To date, the evaluation of BMP on PLGA scaffolds in marginal mandibular defects in the rat mandible has not been described. The present study seeks to determine and optimize BMP-2 dosing in a non-healing critical-sized marginal mandibular defect in a rat model with a PLGA scaffold. We hypothesize that a critically sized marginal mandibular defect can be completely healed through delivery of BMP-2 with PLGA scaffolds and that there exists an optimal physiologic dose of BMP-2, which maximally heals bony defects.

Materials and Methods

Experimental design

Forty-four Sprague-Dawley rats were divided into eight groups to investigate *in vivo* mandibular regeneration of a marginal defect using PLGA scaffolds loaded with BMP-2 (See **Table I** and **II**). In order to determine the lowest dose of BMP-2 necessary to regenerate the marginal mandibular defect, an additional thirty Sprague-Dawley rats were divided into five treatment groups of decreasing BMP-2 (See **Table I**). Additionally, one of the groups of rats was devoted to a microsphere preparation with 0.5 μ g BMP-2 on the PLGA scaffold as well as 0.5 μ g BMP-2 encapsulated in microsphere allowing for release of the additional 0.5 μ g over the course of three weeks (Lee 2007) in order to determine if manipulation of the kinetics of the BMP-2 release had an impact on bone regrowth (See **Table II**).

Surgical treatments consisted of creation of 5 $\times$ 5-mm marginal defects with implantation of a PLGA scaffold loaded with a growth factor. Animals were allowed to heal for eight weeks and then mandibular regeneration was evaluated by microcomputed tomography (microCT) and histology.

Materials

Poly(lactic-co-glycolic acid) (PLGA, lactide:glycolide ratio 85:15, intrinsic viscosity 0.61dL/g) was purchased from Birmingham Polymers (Birmingham, AL). Chloroform (C2432), and methanol (M3641) as well as the growth factor, BMP-2 (B3555), were obtained from Sigma (St. Louis, MO).

Scaffold creation

Apatite-coated PLGA scaffolds were created from 85:15 poly(lactic-co-glycolic acid) (inherent viscosity = 0.61 dL/g, Birmingham Polymers) through a previously described solvent casting/particulate leaching process (Levi). In brief, PLGA/chloroform solutions were mixed with 200-300 μ m diameter sucrose to obtain 92% porosity (volume fraction), and compressed into 5 $\times$ 5 $\times$ 3-mm Teflon molds. After allowing specimens to freeze-dry overnight, the scaffolds were then submerged in three changes of distilled H₂O to wash away the sucrose and freeing the scaffold from the Teflon mold. After this leaching process,

all scaffolds were disinfected by immersion in 50%, 60% and 70% ethanol for 30 minutes each followed by rinses of distilled H₂O. Apatite coating was achieved by incubating scaffolds in simulated body fluid (SBF) as described previously. All scaffolds were then allowed to dry under a laminar flow hood. For protein-loaded scaffolds, proteins were adsorbed by dropping the protein solution onto scaffolds for 20 min followed by further lyophilization in a freeze drier (Labconco; Kansas City, MO). Protein-loaded microspheres were prepared by a double emulsion solvent evaporation method and incorporated into PLGA scaffolds as described previously (Lee 2007).

Animals and procedures

All animal protocols were approved and overseen by the animal research committee at UCLA. The UCLA facility is an AALAC-accredited facility. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animal of the National Institutes of Health. Scaffold regenerative abilities were tested in a previously established (Issa 2008) marginal mandibular critical-size defect in 12-week-old Sprague-Dawley rats. Briefly, the animals received inhalational isoflurane until deep anesthesia was achieved. Critical-sized defects of the mandible are defined as those that will not heal spontaneously during the expected natural life of the animal. The animals were then shaved on the ventral surface of the mandible and prepped and drape in a sterile fashion. Using aseptic technique, an incision overlying and paralleling the left mandible was carried down through subcutaneous tissues. The inferior border of the mandible was then exposed by dividing the pterygomasseteric sling with electrocautery. The lingual and buccal surfaces of the mandible were then exposed through a suprapariosteal elevation of the musculature. Using a high-speed cutting burr at 3,000 RPM with copious irrigation, a 5 × 5-mm square of bone was removed posterior to the incisor and contiguous with the inferior border of the mandible (see **Fig. 1**). Hemostasis was then achieved with electrocautery and the appropriate treatment scaffold is secured within the defect with resorbable sutures (Vicryl 4-0; Ethicon, Inc.) to surrounding soft tissue. The pterygomasseteric sling was then reapproximated with the same resorbable suture and the skin was also closed with non-resorbable nylon sutures. Rats were then allowed to recover from anesthesia and transferred to the vivarium for postoperative monitoring. Postoperatively, all animals received analgesia with subcutaneous injections of buprenorphine (0.1mg/kg) for 72-hours postoperatively. All animals also received trimethoprim-sulfamethoxazole in the water supply for one week following the operation as prophylaxis against infection.

MicroCT analysis

Animals were sacrificed after eight weeks and mandibles were harvested for analysis. Specimens were fixed in 10% formalin for 48 hours, and rinsed in PBS prior to imaging with high-resolution microCT (μ CT40; Scanco USA, Inc., Southeastern, PA). MicroCT data were collected at 50 kVp and 160 μ A. Visualization and reconstruction of the data were performed using Dolphin 3D (Dolphin Imaging & Management Solutions, Chatsworth, CA). Volume analysis of new bone growth was performed using CTAn (Skyscan, Kontich, Belgium). The microCT threshold of 230 was utilized for analysis of mineralization of the mandibular defects. Volume analysis of the specimens included investigations of three volumes and two ratios: defect volume (DV), tissue volume (TV), bone volume (BV), bone

volume/tissue volume (BV/TV), and tissue volume/defect volume (TV/DV). The DV is set as the volume of the original surgical defect (see above) that measured $2.5 \times 5 \times 5$ -mm and is the same across all samples. TV is the amount of new tissue growth (bone and soft tissue) within the defect volume that also includes overgrowth of bone and tissue beyond the original surgical defect when present. BV/TV represents the percentage of new tissue growth that is bone as determined by thresholding. TV/DV represents the percentage of the volume of the defect that was healed.

Histology

Ten- μ m-thick paraffin sections (microtome; McBain Instruments, Chatsworth, CA) of decalcified samples were stained with hematoxylin and eosin according to standard protocols (Cowan 2004). A single sagittally cut slide was taken from the middle of the marginal defect from randomly selected samples within each experimental group in order to confirm presence of compact bone. The slides were then examined with light microscopy.

Statistical Analysis

Bone healing measures (% defect healed, % bone present, bone volume healed) were compared between groups using two-sample t-tests for two groups and one-way analysis of variance (ANOVA) for more than two groups. Kruskal-Wallis non-parametric analysis of variance by ranks was used instead of standard ANOVA in cases where the ANOVA model assumptions were not met (non-normality and homoscedasticity). When the overall ANOVA tests were significant ($p < 0.05$), Tukey's tests and confidence intervals were calculated (with family-wise error rate set at 5%) for differences in means between pairs of groups. All statistical analyses were conducted using SAS software (version 9.2; SAS Institute. Cary, NC).

Results

Animals and Clinical Observations

A total of 44 rats underwent successful creation of a marginal mandibular defect in the left mandible. All animals developed some degree of postoperative swelling at the incisional site that resolved within one week of the operation with no functional consequences. Animals were allowed to heal 8 weeks and then sacrificed for analysis. Only one of the 44 rats (2.3%) developed an abscess and was sacrificed and excluded from analysis.

Healing of Marginal Mandibular Defects

To analyze the ability of BMP-2 to induce bone regeneration *in vivo*, rat 5×5 -mm marginal mandibular defects were implanted with control or varied doses of BMP-2 protein-coated PLGA scaffolds and analyzed at 8 weeks via microCT. PLGA is a common synthetic polymer with an established safety record in humans and not considered osteoinductive and thus allowing for direct comparison of the impact of the growth factors on healing of the defects (Frazza 1971). The current experiment used decreasing doses of BMP-2 (15, 5, 1, 1 with 50% in microspheres and 0.5 μ g) per defect.

To quantitate the extent of BMP-2 induced bone regeneration, new bone volume was investigated on post-mortem microCT analysis. Analysis of mandibular defects demonstrated a trend toward increasing healing of the defect volume with increasing doses of BMP-2 with 15 μ g showing increased healing over all other doses ($p < 0.01$, **Fig. 2** and **Table I**). At a dose of 5 μ g BMP-2 complete defect healing was achieved (see **Fig. 2**, **Fig. 5** and **Fig. 6A**). Closer inspection revealed that even a cortex of bone regenerates at this low dose (see **Fig. 5**). At a dose of 15 μ g BMP-2 there is overfill of the original defect (see **Fig. 6B**). Further analysis of the percent volume healed demonstrates that with increasing doses of BMP-2 there is actually a trend towards a decrease of bone tissue with 15 μ g demonstrating a smaller percentage bone than 0.5 μ g and blank scaffolds ($p < 0.01$, see **Fig. 2**). The total bone formed did not increase significantly beyond the low dose of 5 μ g of BMP-2 (see **Fig. 3**).

Effect of Microspheres on Healing

PLGA has been used as a carrier of microspheres to lengthen the sustained release time of drugs and growth factors (Zou and Peng). In the present study we used a combination of 0.5 μ g of free BMP-2 on PLGA scaffolds as well as 0.5 μ g of BMP-2 encapsulated in microspheres for a total dose of 1 μ g and compared healing of marginal mandibular defects. The total tissue volume healed was significantly higher in the scaffolds that did not include BMP-loaded microspheres (see **Fig. 4**; **Table II**, $p = 0.037$). However, the healed tissue had a significantly higher percentage of bony healing (BV/TV) (see **Fig. 5**, $p = 0.015$).

Histological Analysis

Sampled specimens demonstrated presence of compact bone correlating to distribution of bone seen in microCT data for BMP-2 samples at doses of 5 μ g and 15 μ g. **Figure 5** shows sample slides demonstrating formation of cortical bone at 5 μ g that is better defined than at 15 μ g. **Figure 5** also demonstrates lipid vacuoles within the healed defect.

Discussion

BMP-2-Coated Scaffolds Heal Rat Marginal Mandibular Defects

Graft-based bone regeneration is commonly used to repair mandibular defects caused by various mechanisms, including trauma, tumor resection, osteoradionecrosis and congenital malformations. Reconstruction of critical-sized mandibular defects is a challenging and significant problem that currently requires an invasive harvest of autologous bone, and carries with it the risk of chronic donor site morbidity in up to 11% of patients (Silber). Use of manufactured biodegradable scaffolds with growth factors offers an exciting alternative to the current gold standard. BMPs have been shown to have extraordinary osteogenic potential on a variety of carriers (La); however, to date, the rat marginal mandibular defect has yet to be healed by a BMP-laden PLGA scaffold.

The objective of this study was to evaluate the ability of PLGA scaffolds with varying doses of BMP-2 to heal marginal mandibular defects as an alternative to autogenous bone grafting. BMP-2 is perhaps the most studied osteoinductive growth factor and is currently FDA-approved for bone healing in spinal fusion, maxillary sinus augmentation, and localized

alveolar ridge defects (Infuse, Medtronic Inc.). Some reports are beginning to emerge of off-label use to reconstruct mandibular defects in humans (Clokier, Glied, Herford, Moghadam). Despite significant study in the axial skeleton and long-bone regeneration, there is a dearth of mandibular regeneration studies. The rat serves as a relatively efficient mandibular defect model for marginal defects with the use of a PLGA . Additionally, the impact of dosing of BMP-2 has not been investigated to determine the minimal dose that will significantly induce bone formation in a mandibular defect model (Arosarena 2005).

At a dose of 5 μ g BMP-2, PLGA scaffolds demonstrated complete healing of the defect with evidence of cortical bone development (See **Fig. 2**, **Fig. 5** and **Fig. 6A**) In fact, higher doses of BMP-2 are not needed as they lead to excess bony formation with a decrease in the percentage of bone filled defect (See **Fig. 2** and **Fig. 6B**). The successful restoration of bony mandibular defects using BMP-2-laden PLGA scaffold demonstrated in the present study carries great promise as a viable reconstructive alternative for patients suffering from craniofacial defects.

BMP-2 induces cellular chemotaxis, proliferation, and osteogenic differentiation of both osseous and nonosseous mesenchymal cells (Ahrens, Katagiri). Increasing doses of BMP-2 successfully generated osseous regeneration, but also triggered non-osseous growth in our model. Histological analysis demonstrates, that the tissue present in the overfilled defects contains large lipid vacuoles consistent with adipocyte stimulation (See **Fig. 5**). As we have shown here, increasing doses of BMP-2 generated more tissue growth in our animal, but not necessarily more bone growth. In the present study, our data suggests that in the rat marginal mandibular defect an ideal dose exists at 5 μ g. At this dose, the defect is regenerated completely, without exuberant adipogenic stimulation (see **Fig. 2** and **Fig. 3**). In addition to the excessive tissue production seen with higher doses of BMP-2, the quality of the bone also appears to be affected. Our histologic sections and microCT analysis demonstrates that cortical bone is formed at a 5 μ g dose, but loses definition at a higher dose of 15 μ g (See **Fig. 5** and **Fig. 6**). This data highlights the importance of establishing precise doses for species, site and volume-specific defects when using BMPs, as well as the need for a more bone-specific growth factor. As this study demonstrates, by simply achieving a more physiologic presence of BMP, a more precise and higher quality regeneration can be achieved.

Our study investigated whether the rate of distribution of BMP-2 impacted the quantity and quality of bone regeneration in this animal model. It is reasonable to assume, that if high doses of BMP-2 lead to exuberant growth of non-osseous cells, that a sustained “ideal” BMP-2 dose may produce improved regeneration of bone. Use of growth factors encapsulated in microspheres embedded in PLGA scaffolds has been shown to prolong the release of the growth factor over the course of weeks (Tang). Double-walled microspheres can further provide low steady release of growth factors entirely avoiding the initial supraphysiologic burst seen with other carriers (Zheng). In the current study, when 50% of the dose of the BMP-2 was incorporated into microspheres there was an increase in the percentage of bone within the healed parts of the defect based on volumetric microCT analysis (BV/TV) (See **Fig. 4**). However, despite an increase in quality of regenerated bone produced when using this method, a smaller fraction of the defect was healed. It is possible that the higher density

bone translates to a higher functional capacity. A scaffold can also be further modified to promote bone healing. The present study used apatite-coated PLGA scaffolds as this coating has been shown to promote osteoblastic differentiation and bone formation (Chou; Cowan, Aghaloo 2007). Future studies investigating the impact of the release kinetics of growth factors and carrier attributes hold great promise in elucidating these factors contributions to bone regeneration.

Summary

The utilization of BMPs to restore critical-sized osseous defects of the mandibles carries great promise as an alternative therapy for patients with this problem. The present study demonstrates that our novel rat marginal mandibular model can be successfully healed with low dose BMP-2-loaded PLGA scaffolds. Further analysis demonstrates that the quantity and quality of bone regeneration can be significantly manipulated through adjustment of the dosing and release rate of BMP-2. The testing of novel bone-specific growth factors is critical to advancing our understanding of mandibular regeneration. Similarly, further studies are required to clarify the impact and significance of BMP-2 release kinetics as the current study demonstrates promising, but preliminary data on the use of microspheres to manipulate bony regeneration. An improved understanding of the growth factors and the physiology of bone regeneration in clinically relevant animal models will greatly improve the long-term success by which patients with craniofacial defects may be predictably and effectively treated. This technology has the potential to spare patients the significant medical risks associated with current techniques of mandibular reconstruction and autogenous bone grafting.

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Figure 1.

Surgical creation of a 5×5 -mm marginal mandibular defect based on the inferior border of the mandible was made with a high-speed cutting burr with copious irrigation (A). A PLGA scaffold with varying doses of BMP-2 (B) was then placed into the defect (C) and the pterygomasseteric sling was closed over the defect and the skin over the muscle.

Figure 2.

A histogram displaying the percentage of the defect healed (TV/DV) and the percentage of bone of the new tissue (BV/TV) for controls and increasing doses of BMP-2. There appeared to be an increasing trend in means with 5 µg higher than 0.5 µg ($p < 0.01$) and 15 µg significantly higher than all other dosing for percentage defect healed ($p < 0.01$ for 15 µg vs blank, 0.5 µg, 1 µg and 5 µg on Tukey's comparisons). Similarly, there appeared to be a trend towards decreasing percentage of bone within the healed tissue seen with increasing BMP-2 doses with 15 µg demonstrating significantly less bone percentage than controls and 0.5 µg BMP-2. (** = $p < 0.01$ on Tukey's comparisons)

Figure 3.

A histogram displaying the absolute bone volume formed. One-way ANOVA overall p-value = 0.0002. Tukey's pairwise comparisons demonstrates 5 µg BMP-2 created significantly more bone than 0.5 BMP-2 and the blank scaffold ($p = 0.008$ and $p < 0.0001$, respectively). (** = $p < 0.01$ on Tukey's comparisons)

Figure 4.

A histogram comparing 1 µg BMP-2 on a PLGA scaffold to 0.5 µg BMP-2 on a PLGA scaffold with an additional 0.5 µg BMP-2 encapsulated in microspheres. There was significantly more healing in the unencapsulated dose of BMP-2 compared to the microsphere formulation ($p = 0.037$). However, the percentage of bone filled within the tissue was significantly higher in the microsphere formulation ($p = 0.015$). (* = $p < 0.05$ on Tukey's comparisons)

Figure 5.

Sample histology of healed defects for BMP-2 5 µg (A) and 15 µg (B) dosing. Note the well-developed cortex in the lower dose of BMP-2 (A, white arrow) as compared to the more irregular cortex seen in the higher dose of BMP02 (B, white arrow). Also note the adipocytes (Adp) within the cortices.

Figure 6.

(A) A representative 3D reconstruction of a microCT of the region of interest of the lingual side of a rat mandible regenerated with a PLGA scaffold with 5 μ g of BMP-2. (B) A representative 3D reconstruction of a regenerated marginal defect using 15 μ g of BMP-2 on a PLGA scaffold. Note the bone regeneration beyond the inferior border of the mandible overfilling the original defect.

Table I**Marginal Mandibular Defect Healing Descriptive Statistics**

Scaffold	n	DV (mm ³)	BV (mm ³)	TV (mm ³)	BV/TV (%)	TV/DV (%)
		$\bar{x}$ (SD)	$\bar{x}$ (SD)	$\bar{x}$ (SD)	$\bar{x}$ (SD)	$\bar{x}$ (SD)
No Scaffold	3*	62.8 (0.0)	14.9 (5.3)	20.1 (11.1)	79.0 (17.5)	32.0 (17.8)
Blank Scaffold	11	62.8 (0.0)	11.3 (3.8)	16.5 (6.8)	71.1 (21.8)	26.3 (10.9)
BMP-2						
0.5 µg	6	62.8 (0.0)	13.4 (4.4)	20.0 (5.7)	67.7 (11.7)	31.8 (9.1)
1 µg	6	62.8 (0.0)	15.0 (4.3)	26.4 (7.3)	56.9 (8.4)	42.1 (11.6)
5 µg	9	62.8 (0.0)	20.9 (2.2)	44.1 (11.8)	50.1 (12.8)	70.3 (18.8)
15 µg	3	62.8 (0.0)	17.2 (5.2)	84.0 (25.5)	22.8 (11.3)	133.9 (40.6)

DV = defect volume, TV = tissue volume, BV = bone volume,

* = one animal excluded due to abscess formation

Table II

Effect of BMP-2 In Microspheres on Defect Healing

Scaffold	n	<u>BV</u> (mm ³)	<u>TV</u> (mm ³)	<u>BV/TV</u> (%)	<u>TV/DV</u> (%)
		$\bar{x}$ (SD)	$\bar{x}$ (SD)	$\bar{x}$ (SD)	$\bar{x}$ (SD)
1 μ g (50% in microspheres)	6	18.1 (4.4)	12.9 (1.5)	72.9 (10.4)	28.8 (7.0)
1 μ g	6	26.4 (7.3)	15.0 (4.3)	56.9 (8.4)	42.1 (11.6)

DV = defect volume, TV = tissue volume, BV = bone volume