Pre-augmentation soft tissue expansion improves scaffold-based vertical bone regeneration – a randomized study in dogs

Placement of endosseous dental implants often requires the reconstruction of lost bone. The principles of guided bone regeneration (GBR) include the maintenance of regenerative space and the stabilization of a blood clot due to the placement of scaffolds consisting of granular material and covering barrier membranes on the local bone. The membrane secludes the scaffold from the periosteum and other soft tissues, and angiogenic and osteogenic cells proliferate from the local bone marrow into the membrane-protected area, with the result of intramembranous ossification [Buser et al. 1996; Kostopoulos & Karring 1995; Kostopoulos et al. 1994; Schenck et al. 1994].

As yet, the routine use of GBR is limited to self-containing defects, where remaining bony walls exert mechanical support and protect the scaffold against macro-motion caused by soft tissue movement [Dahlin et al. 1988]; that is, GBR may be primarily used for the regeneration of defects within the contour of residual bone [Esposito et al. 2009; McAllister & Haghighat 2007]. In contrast, the outcomes of extra-skeletal osseous reconstructions appear dubious. Sparse clinical and histological data support the feasibility of extra-skeletal augmentation especially with autogenous bone used as block grafts or with reinforced scaffolding constructs [Esposito et al. 2009; Rocchieta et al. 2008]. Clinical data show a high incidence of soft tissue wound dehiscences with the subsequent exposure of bone grafts or scaffolds [up to 50%], which may cause complete failure of the reconstructive treatment [Jensen & Terheyden 2009; Kaner & Friedmann 2011].
Primary wound closure after placement of grafts or scaffolds for vertical augmentation requires large-scale mobilization and advancement of soft tissues in order to cover the reconstructed area (Greenstein et al. 2009). Dehiscences occur mainly after difficulties in attaining primary wound closure, and due to high strain on the margins of the covering mucoperiosteal flaps (Burkhardt & Lang 2010). Further, flap surgery affects perfusion and induces ischaemia, while ample blood flow is essential for the survival of tissue (Nakayama et al. 1982). Correspondingly, traumatic surgery massively reduces blood supply and causes ischaemia/reperfusion injury, leading to tissue damage and necrosis of the operated tissue (Carroll & Esclamado 2000). Indeed, the extent of surgical trauma and the resultant impairment of soft tissue microcirculation are correlated (Retzepi et al. 2007), while the maintenance of sufficient blood supply to the soft tissues is one of the prerequisites for bone healing (Stegen et al. 2015). Therefore, quality and quantity of soft tissue and its resilience against surgical trauma may be crucial for successful bone regeneration. Preservation of reconstructed bone is another matter of importance, as up to 70% of grafted bone may be resorbed throughout healing (McAllister & Haghighat 2007; Simon et al. 2000). Again, compromised vascularization and soft tissue deficiencies causing the mechanical instability of the augmented area may contribute to these negative outcomes of bone regeneration (Lundgren et al. 2008; Rothamel et al. 2009).

Soft tissue expansion (STE) improves tissue quantity and quality in order to facilitate the subsequent reconstructive surgery, that is, STE provides a tissue surplus for easier primary closure and improves flap vascularity and viability (Asa’ad et al. 2015; Bascom & Wax 2002; Cherry et al. 1983). Pre-treatment by STE prior to vertical augmentation with autogenous bone leads to high gain of new bone, minimizes graft resorption, and results in a very low incidence of wound dehiscences (Abrahamsson et al. 2012; Kaner & Friedmann 2011; Mertens et al. 2013, for review, see Asa’ad et al. 2015). As yet, the effects of STE on the outcome of vertical augmentation have only been investigated using autogenous grafts. Many clinicians prefer autogenous bone over alloplastic materials for complex osseous reconstructions because of its combined osteoconductive and osteoinductive properties (Miron & Zhang 2012). However, harvesting of bone causes significant morbidity and costs, and poses additional risks (Nkenke & Neukam 2014). While alternatives to autogenous bone appear desirable, data on vertical augmentation using GBR with alloplastic scaffolds alone after preconditioning with STE have not yet been published. Here, we report on the effects of STE on GBR using alloplastic scaffolds in an animal model of vertical augmentation.

The hypothesis was that preconditioning with STE would reduce the impairment of perfusion and improve the healing of soft tissues after vertical augmentation; finally, the better preconditioning would improve the outcome in terms of an increased formation of new bone.

Material and methods

The study protocol was in accordance with the European Communities Council Directive of 24 November 1986 [86/609/EEC] and local laws and regulations. It was approved by the Food Safety and Animal Health Protection Board of the Regional Council of Pest/Hungary. The study was carried out at the Research Institute for Animal Breeding and Nutrition, Herceghalom/Hungary, which provided ten male beagle dogs (mean age 8.1 ± 0.9 months, mean weight 12.2 ± 1.3 kg). The dogs were kept in pairs in kennels of five sqm with straw bedding and elevated wooden resting places and were walked twice daily for at least 30 min. They were fed commercially available dog food (Bonafarm, Nagyvajmán/Hungary), and water was provided ad libitum. Surgeries were carried out in general anaesthesia; ketamine hydrochloride (1 ml/10 kg; Algopyrin, Sanofi-Aventis, Frankfurt/Germany) was administered intramuscularly and xylazine hydrochloride (1 ml/10 kg; Xylavet 2%, Sanofi-Aventis, Berlin/Germany) and xylazine hydrochloride (1 ml/10 kg; Algopyrin, Sanofi-Aventis, Budapest/Hungary) were administered intravenously every 15 min. For pain control, intramuscular injections of metamizole (1 ml/10 kg; Algoxyrin, Sanofi-Aventis, Budapest/Hungary) were administered for 3 days. Amoxicillin hydrochloride (150 mg, 1 ml/10 kg, Pfizer, Berlin/Germany) was injected intramuscularly for prophylaxis of infections.

Bone defects and STE

The surgical procedures have been specified earlier in detail (Kaner et al. 2014). Briefly, all premolars were extracted and the tooth-supporting bone was additionally reduced in order to create supra-alveolar critical-size defects on both sides of the mandible (Wikesjo et al. 2006). Six weeks later, tissue expanders [cylinder 0.7 ml; Osmed, Ilmenau/Germany] were placed in submucosal pouches in randomly allocated mandibular test sites for a 5-week expansion phase, according to a split-mouth design, opposing mandibular sides were used as controls (Fig. 1a, b).

Vertical augmentation

0.5 ml of a local anaesthetic [articaine 4% with 1 : 100 000 epinephrine, UDS forte, Sanofi-Aventis, Frankfurt/Germany] was injected each buccally and lingually. At STE sites, the tissue expanders were removed after placement of a mid-crestal incision without releasing incisions. The tissue below the expander was cut in mesiodistal direction, buccal and lingual flaps were elevated, and the bone was exposed.

A similar incision was placed at control sites. Additional releasing incisions were extended from the canine and the first molar into the mucosa. Buccal and lingual flaps were raised, and the bone was exposed.

The recipient bone at test and control sites was perforated to provoke bleeding, and the defect was augmented with a scaffold consisting of granular biphasic calcium phosphate soaked with blood, and a resorbable polyethylene glycol membrane (BCP/PEG; Institut Straumann AG, Basel/Switzerland). Length, width and height were measured in millimetres, and the volume of the semioval scaffold was calculated in mm³ (ScVol). Wound closure at test sites was attained without additional flap advancement. At control sites, periosteum and submucosa were stripped and dissected until the flaps covered the scaffold passively, as recommended (Greenstein et al. 2009). Flaps were closed with vertical mattress sutures and fine
continuous sutures [Vicryl 3.0 and Monocryl 6.0, Ethicon, Norderstedt/Germany].

Microcirculation and soft tissue healing
Results on microcirculation and soft tissue healing have been reported previously in detail [Kaner et al. 2015]. The primary outcome, soft tissue dehiscence [yes/no], was evaluated after 2 weeks. Flap microcirculation was measured at augmentation surgery before local anaesthesia [baseline], 2 min later, immediately after surgery, and after 3 days, using laser Doppler flowmetry (LDF; Periflux 5010, PF 416 probe, outside diameter 1.0 mm, fibre separation 0.25 mm, wavelength 780 nm, Perimed AB, Jarfalla/Sweden).

Preparation of specimens
After 8 weeks, all animals were killed with pentobarbital. The mandibles were dissected and snap-frozen at −80°C. Later, the samples were thawed in buffered 4% formalin for 22 h. The specimens were dehydrated in graded ethanol and xylene, and infiltrated and embedded in resin (Technovit 9100, Heraeus Kulzer, Wehrheim/Germany) at −15°C. All specimens were sectioned in buccolingual direction into slices of 80 μm thickness, using a saw microtome [SP1600, Leica, Bensheim/Germany], and stained [Masson Goldner trichrome]. Digital images were obtained using a light microscope equipped with a digital camera with 5-megapixel CCD [Leitz DMRB and Leica DFC 425 C, Leica, Wetzlar/Germany].

Histomorphometry
The total augmented area (TA) in each specimen was determined by agreement between three investigators [AF, HZ and WA]. Single images of the augmented area under 32-fold magnification were stitched to an overview image, using image-processing software [Adobe Photoshop CS6, Adobe, Dublin/Ireland]. One investigator (HZ) calculated areas for new bone (NB), osteoid (O), connective tissue (CT) and residual particles (RP), and their proportions [%] of TA, using AutoCAD 2011 software [Autodesk, Munich/Germany].

Immunohistochemistry
The sections were treated with 2-methoxyethyl acetate for 4 min to dissolve the resin and were rehydrated in graded alcohol and washed in PBS for 10 min. Endogenous peroxidase was blocked with peroxidase block (Dako, Hamburg/Germany) for 5 min. Antigens were unmasked using trypsin [TG-II: Abcam, Cambridge/UK] and proteinase K (OC: Abcam, Cambridge/UK), respectively, for 10 min. Non-specific binding was blocked by incubation with blocking solution [Danko, Hamburg/Germany] for 15 min. The sections were incubated with primary antibodies [TG-II: anti-transglutaminase-II AB-1, mouse antibody, Lab Vision Corporation, Fremont/CA, USA; OC: anti-osteocalcin, mouse antibody, Abcam, Cambridge/UK], respectively, for 50 min. Then, the slides were washed in PBS for 10 min and incubated with streptavidin-peroxidase solution [Dako, Hamburg/Germany] for 45 min. 3-Amino-9-ethylcarbazole [Dako, Hamburg/Germany] was used as chromogen. A semiquantitative evaluation of staining intensity [negative/weak/strong] was independently carried out by three investigators [WA, AF and DK].

Statistical analysis
One investigator [DK] carried out all statistical analyses. Sample size was calculated for the primary endpoint [soft tissue dehiscence, binary outcome yes/no] with the animal as the unit of analysis [Rosner 2010]. The assumption that the proportions of dehiscences would be 50% in control sites and 0% in test sites resulted in a sample size of nine animals in a split-mouth design [two-sided test, α = 0.05, β = 0.8]. In order to compensate for potential dropouts, ten dogs were used.

Medians and quartiles were calculated for ScVol, changes in LDF measurements and histomorphometric parameters and their proportions of TA. Mann–Whitney U-test and Fisher’s exact test were used for comparisons between test and control groups. Wilcoxon’s signed-rank test was used for longitudinal comparisons within groups. Correlations between histomorphometric parameters were analysed using Spearman’s correlation coefficient. Levels of TG-II and OC were compared using the chi-squared test. The level of significance was α = 0.05. All histomorphometric and immunohistochemical evaluations and the statistical analyses were carried out blinded to the group allocation.

Results
Expander surgery and initial healing were without complications. Later, three expanders were lost before augmentation, as reported previously in detail [Kaner et al. 2015]. At augmentation surgery, similar-sized scaffolds were applied in both groups [test: median ScVol = 141 mm³ [IQ 110; 191], control: median ScVol = 130 mm³ [IQ 108; 161], P = 0.529].

Microcirculation and soft tissue healing
Detailed results for microcirculation and soft tissue healing have been reported previously [Kaner et al. 2015]. Uneventful healing in test sites contrasted with the finding of eight dehiscences in control sites (P = 0.002).

Comparable LDF measurements before augmentation and similar declines caused by local anaesthesia were found in both groups [data not shown]. However, perfusion dropped significantly after augmentation surgery in control sites (P < 0.001), whereas test site perfusion remained stable (P = 0.627). Accordingly, microcirculation after augmentation was significantly better in STE sites (P = 0.012).

From the termination of augmentation surgery to the third post-surgical day, no change was found in test sites, while control sites showed significant increases in perfusion (P = 0.001). Nevertheless, microcirculation measurements at control sites were – contrasting to test sites – still significantly lower after 3 days, when compared to baseline (P = 0.005).

Histomorphometry
Total augmented area
Figure 3 depicts representative sections after 2 months of healing. Test sites and control sites showed similar median values for TA.
New bone area

New bone was found in all ten test sites, whereas two of ten control specimens showed no NB formation at all. Test sites showed greater areas of NB than control sites (Table 1). NB covered 3.81 mm² (median, IQ 1.33, 5.35) in test sites, whereas control sites showed a NB area of 1.06 mm² (median, IQ 0.43, 1.39). The difference was statistically significant \( P = 0.023 \) favouring the test group.

Proportion of connective tissue

CT covered 19.80% (median, IQ 10.98%; 46.95%) of TA in test sites, whereas control sites showed a CT proportion of 42.27% (median, IQ 17.29%; 93.39%) of TA (significant difference favouring the test group, \( P = 0.009 \), Table 1).

Other parameters

For NB%, CT, O, O%, RP and RP%, no significant differences were found between both groups [data not shown].

Correlations between histomorphometric parameters

The correlation between the area covered with osteoid and TA was significant in both groups \( r = 0.794, P = 0.006 \); control: \( r = 0.927, P < 0.001 \). Both groups showed similar correlations between TA and the amount of RP \( r = 0.818, P = 0.004 \); control: \( r = 0.830, P = 0.003 \).

However, NB was correlated with TA in test specimens only \( r = 0.794, P = 0.006 \), Fig. 4a. In contrast, control specimens showed no significant correlation between NB and TA \( P = 0.059 \), Fig. 4b.

Discussion

We compared the outcomes of vertical augmentation using GBR between sites with or without preceding STE in a critical-size defect model that has been designed for rigorous testing of techniques and materials aimed at supra-alveolar bone regeneration [Wikesjo et al. 2006]. The volume of the BCP/PEG scaffolds placed on the residual bone was similar in both groups. Histomorphometric sites showed a CT proportion of 42.27% (median, IQ 17.29%; 93.39%) of TA (significant difference favouring the test group, \( P = 0.009 \), Table 1).

Table 1. Histomorphometric parameters for test and control groups (medians and interquartiles)

| Parameter | Test          | Control       |
|-----------|---------------|---------------|
| TA (mm²)  | 9.49          | 5.26          |
| NB (mm²)  | 3.81*         | 1.06*         |
| CT (% of TA) | 19.80†       | 42.27†        |
| IQ        | (4.62, 14.85) | (3.35, 6.97)  |
| Range     | (0.21–8.63)   | (0.43–1.39)   |

\*Significant difference in favour of the test group, \( P = 0.023 \).
†Significant difference in favour of the test group, \( P = 0.009 \) (Mann–Whitney U-test).

TA: total augmented area; NB: new bone; CT: connective tissue.

Table 2. Chi-squared test results

| Parameter | Test | Control |
|-----------|------|---------|
| NB (%)    | 3.81 | 1.06    |
| CT (%)    | 19.80| 42.27   |

\( P = 0.023 \), chi-squared test, Table 2.

Transglutaminase II

In the test group, three specimens were TG-II negative. Two specimens showed a weak staining only, while five specimens showed a strong TG-II reactivity (Fig. 5c). In the control group, six specimens were TG-II negative and four specimens showed a weak staining (Fig. 5d). No control specimen showed a strong TG-II reactivity. The difference between the groups was statistically significant \( P = 0.036 \), chi-squared test, Table 3.

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calculated showed no difference between test and control sites with regard to the total augmented area after 8 weeks of healing. Obvious bone formation and similar amounts of osteoid and residual BCP particles in both test and control sites indicate bone regeneration according to the GBR principle regardless of group allocation, and corroborate the results for these materials obtained in less critical situations such as grafting of the maxillary sinus or of other self-containing defects. In these favourable conditions, osteogenesis around BCP granules resembles intramembranous bone formation, passing the stages of osteoid formation, mineralization, the generation of fibrous bone and remodelling (Friedmann et al. 2009, 2015; Thoma et al. 2012). Likewise, both groups showed a similar immunohistochemical reactivity for the mineralization marker osteocalcin, as found previously in studies that demonstrated the osteoconductivity of BCP in straightforward dehiscence-type defects around implants (Sager et al. 2012; Schwarz et al. 2010).

Nevertheless, test sites with preceding STE showed significantly greater areas of new bone formation. Consistently, significantly higher proportions of connective tissue were found in control sites, and two of ten control sites failed to show any bone formation at all. The significantly stronger immunohistochemical reactivity for TG-II, a marker substance for a maturating bone matrix and ongoing calcification (Nurminskaya & Kaartinen 2006), observed in the bone matrix around residual particles in test sites, matches the histomorphometric outcomes and emphasizes the different outcomes of osteogenesis in both groups.

Inferior bone formation in sites without STE was preceded by poor soft tissue healing after augmentation surgery, when test and control sites are compared. In all STE sites, soft tissues healed primarily and without complications. Conversely, exposure of the BCP/PEG scaffold to the oral cavity was the consequence of soft tissue dehiscence in eight control sites. Premature degradation of membranes, bacterial infection of the surgical site, exfoliation of scaffold material and secondary healing are consequences of graft exposure. Inflammatory infiltrates are common around scaffolding materials in sites after secondary healing, and as found in compromised control sites in our study, bone regeneration may either be limited or fail completely (Moses et al. 2005; Friedmann et al. 2015).

Linear correlations between the total augmented area (TA) and other histomorphometric parameters are to be expected as outcomes of GBR (von Arx et al. 2001); that is, a given scaffold volume should predictably generate a certain amount of new bone. In both groups, TA was indeed positively correlated with the areas covered with osteoid and with residual particles, respectively. Interestingly, a highly significant and strong correlation between TA and the amount of new bone was solely found in STE sites ($r = 0.794$, $P = 0.006$), indicating that a reliable and predictable relation between the scaffold volume and the amount of newly formed bone existed in test sites only. Obviously, the positive effects of STE on soft tissue healing proved beneficial on the outcome of GBR applied for vertical augmentation.

Besides complications, a scaffold’s resilience against deformation and displacement and its capability in maintaining space affect bone healing (Babis & Soucacos 2005; Moses et al. 2005). Mechanical stability is a prerequisite for vascularization and angiogenesis during bone formation and is necessary to allow incorporation and remodelling of implanted scaffolds (Babis & Soucacos 2005; Dimitriou et al. 2011). Likewise, mechanical instability affects the proliferation and differentiation of regenerating tissues: while direct bone formation occurs in regions exposed to neither compressive stress nor tensile strain, high tensile strain and increased macro-mobility increase the formation of fibrous tissues and inhibit osteoneogenesis (Carter et al. 1998; Hiltunen et al. 1998). In our study, the tissue surplus created by STE may have increased the resilience of the BCP/PEG scaffolds against deformation and displacement, as illustrated by higher amounts of new bone

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**Table 2. Immunohistochemical reactivity for osteocalcin (the number of specimens)**

|                | Negative | Weak | Strong |
|----------------|----------|------|--------|
| Test           | 5        | 1    | 4      |
| Control        | 6        | 3    | 1      |

No significant difference between the groups ($P = 0.236$, chi-squared test).

**Table 3. Immunohistochemical reactivity for transglutaminase II (the number of specimens)**

|                | Negative | Weak | Strong |
|----------------|----------|------|--------|
| Test           | 3        | 2    | 5      |
| Control        | 6        | 4    | 0      |

Significant difference favouring the test group ($P = 0.03$, chi-squared test).

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Fig. 5. a, b. Immunohistochemical stainings for OC with strong staining intensity scattered around BCP particles in test specimens [a]. As well, positive staining is found in small zones around the BCP particles in control specimens [b]. No statistically significant difference between groups (Table 2). c, d. Immunohistochemical stainings for TG-II with very strong staining intensity around BCP particles in test specimens (c). Some positive staining is found in small zones around the BCP particles in control specimens (d). The difference between both groups is statistically significant (Table 3).
and the strong correlation between NB and TA.

In order to place a scaffold, a mucoperiosteal flap has to be elevated, and the resultant surgical trauma disturbs perfusion and induces ischaemia [McLean et al. 1995]. The extent of the subsequent microvascular damage can be monitored by LDF [Retzepi et al. 2007]. LDF measurements taken directly after augmentation surgery and after 3 days, respectively, showed distinct differences between sites with and without preceding STE. Augmentation surgery caused a significant additional decrease in microcirculation in control sites, whereas no further reduction in perfusion beyond the effect of local anaesthesia was observed in STE test sites. Accordingly, microcirculation was significantly better in test sites after the conclusion of augmentation surgery, and perfusion had fully recovered after 3 days. In contrast, a significant disturbance of microcirculation was still present in control sites 3 days after surgery. LDF has been used previously to evaluate the effects of differing surgical invasiveness. Minimally invasive surgeries lead to earlier recovery of perfusion than conventional techniques [Retzepi et al. 2007]. Furthermore, arteries traverse obliquely from posterior in canine and human mandibles [Jeffcoat et al. 1982; Kleinheinz et al. 2005], and releasing incisions in mucosa and periosteum basically affect the revascularization of mucoperiosteal flaps [Mörmann & Ciancio 1977]. This is in strong agreement with our findings, which clearly attribute the negative impact of augmentation surgery on microcirculation and wound healing to the standard technique for wound closure applied in control sites without STE: in order to close the flap above the BCP/PEG scaffold, long releasing incisions and incisions into the submucosa were placed, and repeated stripping of the periosteum had to be carried out. Sufficient vascularization in order to allow the influx of stem cells is a precondition for bone healing [Stegen et al. 2015]. Therefore, angiogenesis is essential for successful bone regeneration [Glowacki 1998; Kanclerz & Oreffo 2008]. However, non-pedicled grafts initially obtain nutrients and oxygen through diffusion from surrounding tissues only [Albertius et al. 1996; Winet 1996]. Hence, impairment of perfusion critically affects bone healing [Glowacki 1998; Kanclerz & Oreffo 2008]. Periosteal vessels are important for osseous blood supply, and mobilization and stripping of the periosteum do not only decrease the blood flow in the mucosa, but also reduce perfusion of the outer cortical bone itself [Kowalski et al. 1996].

The effects of STE exceed sole increases in tissue volume and thickness; hence, the good outcomes of reconstructive surgery after STE are not only attributed to the surplus of tissue, but also to its mechanic, vascular and angiogenic properties as well [Bascom & Wax 2002]. Regardless of whether expanders are implanted above or below the periosteum, a dense and mechanically stable connective tissue capsule forms around the device after a retention time of several weeks [Abrahamsson et al. 2009, 2012; Kaner & Friedmann 2011; Tominaga et al. 1993]. The capsule is highly vascularized and improves flap viability [Johnson et al. 1993]. Higher microvessel capacity and blood flow were found in soft tissue above block grafts, when STE had been carried out in advance. In contrast, perfusion of the overlying tissues stops for 10 days, when grafts are placed without preceding STE [von See et al. 2010]. In addition, expanded skin has even been found to express vascular endothelial growth factor (VEGF) [Lantieri et al. 1998]. Further, implantation of silicone expanders or resins like polymethylmethacrylate (PMMA) induces a foreign body reaction that depends on expansion speed, surface properties and retention time [Anwander et al. 2007; Wiese 1993; Wiese et al. 2001]. Implantation of a non-expanding PMMA spacer has been shown to induce the formation of a pseudosynovial membrane that could subsequently be used to enclose bone grafts in continuity defects of long bones. Interestingly, the stimulated membrane did not only express VEGF, but other growth factors and osteoinductive factors such as TGF-β, vWF and BMP-2 as well [Christou et al. 2014; Pelissier et al. 2004].

Following the principle that therapies that improve tissue vascularization likewise improve healing and formation of bone [Stegen et al. 2015], the connective tissue capsule induced by the implanted expander material may have led to additional beneficial effects on the integration of the osteoconductive BCP/PEG scaffolds.

Conclusions

Preconditioning with STE improved microcirculation and soft tissue healing and optimized the mechanical and biological conditions for bone formation in the applied animal model of vertical augmentation using GBR with alloplastic scaffolds. The good outcome of bone regeneration using the combination of STE and GBR may reduce the need for harvesting of autogenous bone in complex augmentation procedures.

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