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Histomorphometric assessment of bone formation in sinus augmentation utilizing a combination of autogenous and hydroxyapatite/biphasic tricalcium phosphate graft materials: at 6 and 9 months in humans

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Abstract

Objective: The aim of this study was to examine the efficacy of a new biphasic hydroxyapatite/tricalcium phosphate (HA/TCP) bone substitute in combination with particulate autogenous bone in sinus floor augmentation procedures.

Material and methods: A simultaneous or a two-stage sinus augmentation and implant placement were conducted in 28 patients. A mixture of HA/TCP and autogenous bone chips in a 1:1 ratio was used as the grafting biomaterial. Cylindrical specimen bone retrieval was performed in all patients except one. Specimens were harvested either at 6 ($n = 14$) or 9 ($n = 13$) months post-augmentation. For histologic and histomorphometric evaluations, the non-decalcified tissue processing (Donath's technique) was performed.

Results: Newly formed bone around the grafted particles was found in all samples. The encircling, highly cellular bone followed the outline of the grafted particles in direct contact. Both woven and lamellar types of bone were observed.

Morphometrically, the total mean bone area fraction of all sections was $34.8 \pm 10.3\%$, increasing from $28.6 \pm 7.8\%$ at 6 months to $41.6 \pm 8.3\%$ at 9 months ($P < 0.001$). Mean particle area fraction average was $25.5 \pm 11.6\%$ and $23.5 \pm 9.3\%$ at 6 and 9 months, respectively, with a total mean of $24.5 \pm 10.4\%$. The increase in bone area fraction was not significantly correlated to the decrease of the grafted particles area fraction.

Conclusions: The biphasic HA/TCP showed biocompatible and osteoconductive properties. This alloplast as a composite with autogenous bone chips promotes newly formed bone, which increases in its fraction along an extended healing period.

Autogenous bone of intramembranous origin has been applied as bone augmentation material with excellent results (Misch & Dietsch 1994; Jensen et al. 1998; Misch 1999; Tulasne 1999), and can be harvested in multiple forms, such as particles, strips, or blocks. Autogenous bone harbors osteoinductive and osteoconductive properties, as it contains growth factors and has a scaffold effect for osteogenic transfer. Its immunogenic competence and rapid healing, incomparable with any non-autolo-

gous source, make autologous source the gold standard for bone reconstructive surgery (Burchardt 1983). The key to success of any bone graft is primarily determined by the degree of revascularization.

Nevertheless, the use of autologous bone for augmentation possesses several shortcomings – donor site morbidity, limping when the source comes from the hip, prolonged healing period, and unpredictable graft resorption. Rapid revascularization and complete resorption of autogenous

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bone (Davis et al. 1984) may not ultimately serve its long-term goals, especially in certain augmentation cases, e.g., sinus grafting or certain large alveolar bone defects, where ridge augmentation procedures are indicated. These require a long-lasting osteoconductive guiding scaffold to support *de novo* bone formation. Therefore, a bone substitute added to a particulate autogenous bone, which is biocompatible and osteoinductive, or at least osteoconductive and can accommodate and integrate to load-bearing titanium implants, has become an ultimate demand.

The early meta-analysis of grafted maxillary sinuses (Tong et al. 1998) demonstrates the excellent results obtained by using the composite combination of autogenous and non-autogenous grafting material whether using xenografts or allografts over applying a pure non-autogenous grafting source.

These findings were repeatedly confirmed by research groups in animal (Hürzeler et al. 1997) and human (Watzek et al. 1998; Boëck-Neto et al. 2002; Hallman & Zetterqvist 2004; Artzi et al. 2005; Peleg et al. 2006) clinical trials. Furthermore, this composite combination has shown its efficacy in lateral augmentation of the alveolar ridge (Hellem et al. 2003) and has been tested in a critical-size defect in goats (Merckx et al. 2000). The authors state that the composite graft combines the advantages of each material alone and reduces the disadvantages of each when used separately. However, the use of cancellous bone chips showed a better outcome than the cortical ones.

The advantages of a combined autologous and non-autologous source, which would serve as an inductive vehicle, and also act as a slow resorbable osteoconductive vehicle, would probably be the graft of choice (Laurencin & Lu 1999; Lynch 1999; Temenoff et al. 1999).

Various bone graft materials have been evaluated in sinus augmentation procedures (Jensen et al. 1998; Garg 1999; Kirsch et al. 1999). Both alloplasts and xenografts have shown predictable results (Jensen et al. 1998).

A biphasic hydroxyapatite/ β -tricalcium phosphate (HA/TCP) is a new, bone-graft substitute produced by a single process to prevent clustering and to establish a new homogeneous molecule. Its 60:40 ratio of

HP/TCP gives it two phases of activity. HA/TCP offers an interconnected porosity of 90% (pores ranging from 100 to 500 μ m in diameter) to support cellular penetration.

Rapid attraction of newly formed bone has been shown when HA/TCP-coated implants were examined (Burr et al. 1993; Lee et al. 2001; Stewart et al. 2004). Thus, HA/TCP is recognized as an osteoconductive and bioactive material. The main advantage is that it is an excellent cell carrier, i.e., mesenchymal stem cells to promote bone formation (De Kok et al. 2003; Mankani et al. 2006a; Trojani et al. 2006). Observations *in vivo* (Mankani et al. 2001, 2006b) showed that the best results in tissue engineering, i.e., greatest osteogenesis, were obtained when bone marrow stromal cells were combined with HA/TCP particles. Furthermore, this ceramic alone was sufficient to induce cell differentiation and actually harbored an intrinsic osteoinductive property (Tan et al. 2007). In the socket sites in dogs, a steady increase in bone formation was evident when culture-expanded bone marrow-derived mesenchymal stem cells adherent to HA/TCP were implanted (De Kok et al. 2005). However, in critical-size defects in the rat calvaria, HA/TCP elicited bone only when attached to a macroporous disc (Fleckenstein et al. 2006).

It has been claimed that an optimum balance of the stable phase of HA and the soluble phase of TCP could increase new bone formation, as it releases calcium and phosphate ions into the biological medium (Daculsi et al. 2003). While the compound of HA and biphasic TCP show promising results extra-orally (Russotti et al. 1987; Brook et al. 1991; St John et al. 1993; Emery et al. 1996; Gauthier et al. 2001; Le Nihouannen et al. 2005; Schopper et al. 2005; Blouin et al. 2006; Fella et al. 2006) and intra-orally (Hashimoto-Uoshima et al. 1995; Boix et al. 2004, 2006) in animal studies, it still lacks validation in intra-oral applications in humans. Furthermore, in accordance with the available literature, the use of a composite graft of HA/TCP and autogenous bone graft in sinus floor elevation procedures in human patients has not been evaluated.

The aim of the study was to examine the histology of healing and bone formation pattern at 6 and 9 months, and to evaluate morphometrically, the amount of newly

formed bone, when using HA/TCP mixed with 1:1 particulate autogenous bone chips as the augmentation biomaterial in 28 consecutive sinus grafting procedures.

Material and methods

The study comprised 28 sinus floor augmentation procedures, using either simultaneous or delayed implant placement protocols. Residual bone height, as measured on the serial sections of the computerized tomography (CT) scan in each case, ranged from 1 to 6 mm.

The decision to place implants simultaneously with the grafting procedure or in a two-stage event was determined by the amount of residual bone volume. Implants were placed in conjunction with the bone grafting phase when the CT scan showed 4–6 mm of bone height and adequate bone width. Thus, initial stability of the fixtures was achieved. As implant initial stability could not be assured in 1–3 mm residual bone height, a delayed implant placement phase was decided.

Patients (17 females, 11 males), ranging in age from 31 to 75 years (average 53.2 years), were reported as systemically healthy and there was no requirement for routine medication. Each surgical step was explained and patients signed a consent form. The Ethics Committee of the University approved the study.

A panoramic radiograph and a CT scan of the maxilla were taken pre-operatively for each patient. Antral spaces were evaluated at 2 mm serial sections.

The augmentation biomaterial consisted of a mixture of 1:1 ratio of HA/TCP (Straumann Bone Ceramic[®], Straumann AG, Basel, Switzerland), 500–1000 μ m size particles, and autogenous cortical bone chips harvested from the lateral antral wall and/or posteriorly by a bone scraper (MxGrafter[®], Maxilon Laboratories Inc., Hollis, NH, USA). Blood coagulum was added for moisture.

Premedication followed the protocol suggested by Misch (1999). That is, dexamethasone (Rekah Pharmaceuticals, Holon, Israel) 9 mg pre-operatively, 6 mg after 24 h, and 3 mg after 48 h, as an anti-inflammatory drug. Systemic antibiotics, amoxicillin (Moxypen[®], Teva Pharmaceuticals, Petach Tikvah, Israel), were also

administered 1 h pre-operatively (1 g) and 500 mg (QID) for 1 week. As an analgesic agent, Etodolac 600 mg (Etopan, Taro, Haifa Bay, Israel) initial dose, and 200–400 mg as needed, was also prescribed. The sequential surgical steps are well known and follow the pioneering technique of Boyne & James (1980) and Tatum (1986), previously described in detail (Jensen 1999; Misch 1999; Artzi et al. 2001; Hallman et al. 2001).

Briefly, the lateral window location and boundaries were pre-planned with a CT scan. Once the window was established, the Schneiderian membrane was gently reflected from the inner lateral bony wall aspect by a specialized broad flat curette. Grafting material coagulum was packed in the exposed area of the sinus, between the Schneiderian membrane and the bony floor of the sinus, up to a full completion of the lateral window. The exact location of the window boundaries, i.e., augmentation area, was recorded by a periodontal probe for future harvesting procedure orientation (Fig. 1). The harvesting procedure occurred exactly at the core of the lateral window area in a lateral-inward and upward direction. Thus, no residual bone was involved in the harvested specimen. Careful attention was given to the proximity of the implants in the simultaneous approach.

Before soft tissue closure, the entire obturated lateral window was covered with an absorbable cross-linked collagen membrane (Ossix[®], Colbar LifeScience, Herzliya, Israel).

As an antiseptic mouthwash, a 0.2% chlorohexidine gluconate solution (Tarodent Mouthwash, Taro Pharmaceutical Industries Ltd, Haifa Bay, Israel) was used for 45 s, twice daily for 2 weeks. Sutures



Fig. 1. Measurement of window boundaries taken with a periodontal probe for harvesting procedure orientation.

were removed after 14 days. Soft tissue healing was uneventful, except for one patient who developed post-operative swelling at 4 weeks. Consequently, this patient was omitted from the study. Radiographically, the grafted biomaterial particles were dominant by their marked radio-opacity.

Cylindrical bone samples were harvested with a 2.5 mm internal diameter trephine exactly at the previous location of the lateral fractured window area in a diagonal inward and upward direction (Artzi et al. 2001, 2003, 2005). The ability to determine the exact location of the core area of the lateral window site enables the harvesting of a pure augmented bone specimen without involving residual origin tissues of the host.

This procedure was carried out during the implant placement phase, 6 or 9 months post-augmentation in the two-stage approach, or at the time of implant cover screw exposure phase, at 6 or 9 months, in the simultaneously placed implants with the sinus augmentation procedure. The specimen length varied from 6 to 8 mm.

Histologic preparation

Histologic processing was performed at Biomatech-Namsa Labs, Chasse Sur Rhone, France. Specimens were fixed in 10% neutral-buffered formalin for 1 week followed by a non-decalcification sectioning process, i.e., the Donath technique (Donath & Breuner 1982; Donath 1985; Rohrer & Schubert 1992).

Specimen blocks were dehydrated in increasing concentrations of alcohol, cleared in xylene, and embedded in polymethyl-metacrylate (PMMA) resin (Technovit 7200 VLC, Heraeus Kulzer GmbH Co, KG, Hanau, Germany). Histologic sections were obtained by a microcutting and grinding technique, adapted from Donath (Donath & Breuner 1982). Sections were stained with toluidine blue basic fuchsin (Paragon staining) for qualitative and quantitative histology. Photomicrographs were obtained with a Zeiss Axiolab photomicroscope (Carl Zeiss Microimaging Inc., Thornwood, NJ, USA).

Sections were examined morphometrically using the NOVA Image Analysis Software (BIOQUANT Image Analysis Corporation, Nashville, TN, USA). The entire circumference of the section (con-

taining bone, grafted particles, and connective tissue) was traced manually to create an individual region of interest (ROI), in which the relative area of bone tissue (stained purple) and residual particles (stained black) was measured as percentage of the total ROI area.

Statistical analysis

Data are presented as mean \pm SD. Differences in the mean values of bone and particle area fractions between time groups were analyzed with Levene's test for equality of variances followed by non-paired *t*-tests. Pearson's correlation coefficients between bone and particle area fractions were calculated for the entire sample or for each time point separately.

Results

Only one patient developed sinusitis and swelling post-operatively, which were relieved with prolonged antibiotics. As the sinusitis was exhibited bilaterally as diagnosed by her ENT physician, it was assumed to be coincidental. However, this patient was excluded from the study. Consequently, 27 specimens were retrieved and evaluated.

Newly formed bone was evident around the grafted particles in all specimens (Fig. 2a), and was more pronounced in proximity of the biodegradable membrane. At these stages (6 or 9 months), the established osseous tissue was predominantly surrounding the grafted biomaterial particles and followed its configuration (Fig. 2b).

On high magnification, highly cellular osseous tissue was the predominant form of bone around the particles, occasionally exhibiting irregular margins (Fig. 2c). Many viable osteocytes could be seen within the newly formed bone closest to the grafted particles (Fig. 2d). Using polarized microscopy, some areas of the bone adjacent to the particles displayed lamellar organization (Fig. 3a and b).

Seldom, residues of the overlying cross-linked collagen membrane could be observed (Fig. 4a). In one site, the membrane appeared to be partially calcified based on its staining characteristics (Fig. 4b).

In all 27 samples, the area fraction of the newly formed bone ranged from 17.6% to 53.8%, with an average of $34.8 \pm 10.3\%$.

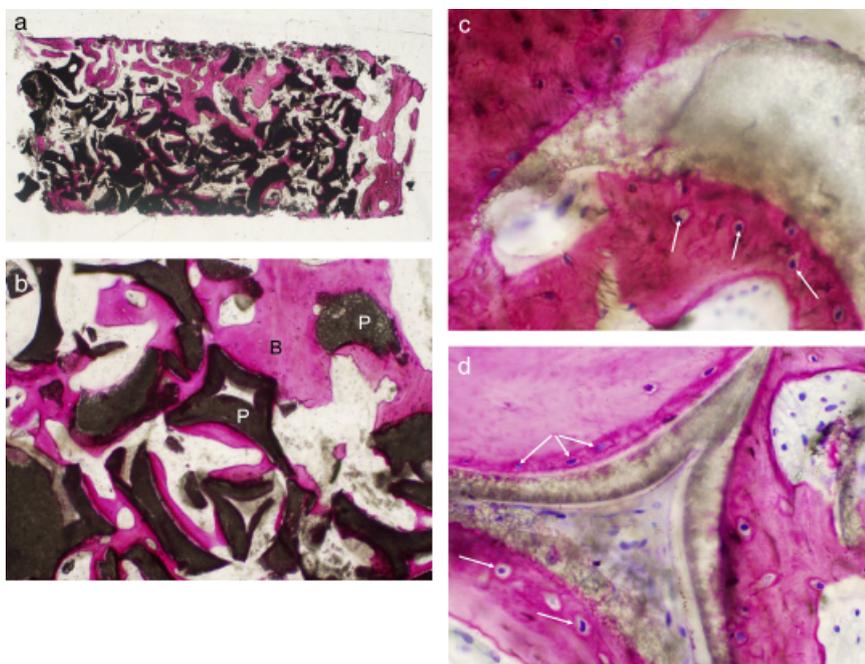


Fig. 2. (a) Typical cylinder tissue core from the augmented site (Paragon staining, original magnification $\times 30$). (b) Most of the grafted particles (P) surrounded by newly formed bone (B) (Paragon staining, original magnification $\times 150$). (c) A HA/TCP particle with irregular margins embraced by highly cellular (arrows) osseous tissue (Paragon staining, original magnification $\times 600$). (d) Osteocytes (arrows) lining the interface osseous zone in direct contact with the grafted HA/TCP particle (Paragon staining, original magnification $\times 600$).

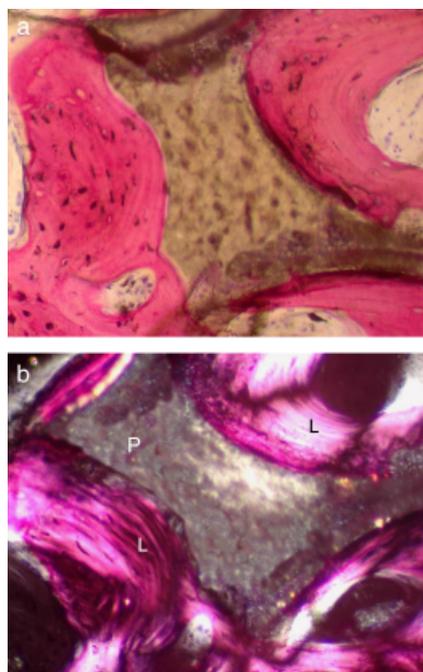


Fig. 3. (a) A high magnification of a 6-month period grafted particle fully surrounded by the newly formed bone (Paragon staining, original magnification $\times 600$). (b) Polarized light image of part (a). An area of bone tissue adjacent to the particle (P) displays lamellar structure (L). (Paragon staining, original magnification $\times 600$).

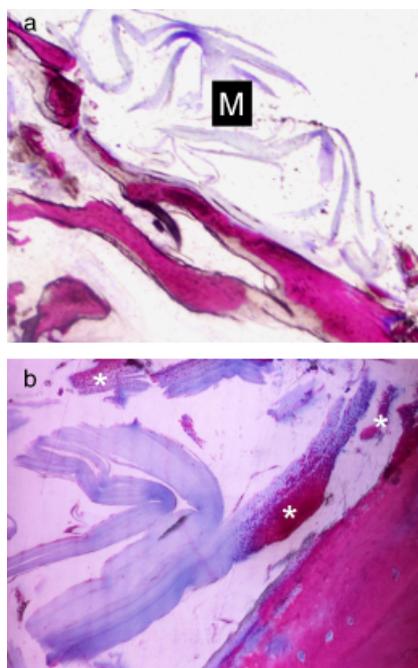


Fig. 4. (a) Remnants of the overlying collagen membrane (M) can be observed (Paragon staining, original magnification $\times 30$). (b) Cross-linked collagen membrane exhibits partial calcification (*) (Paragon staining, original magnification $\times 150$).

The percentage of the grafted particle area ranged from 7.8% to 57.2%, with an average of $24.5 \pm 10.4\%$ (Table 1). Mean bone area fraction increased from $28.6 \pm 7.8\%$ at 6 months to $41.6 \pm 8.3\%$ at 9 months ($P < 0.001$) with no significant difference between the mean particle area fraction at 6 ($25.5 \pm 11.6\%$) and 9 ($23.5 \pm 9.3\%$) months (Fig. 5).

No correlation between bone area fraction and particle area fraction for the 6-month samples ($n = 14$) was found; however, within the 9-month samples ($n = 13$), a trend toward a negative correlation approached statistical significance ($r = -0.47$, $P = 0.09$).

Discussion

The highly porous HA/TCP alloplast proved to be a biocompatible biomaterial and safe. The 50:50 composite of HA/TCP and particulate autogenous bone promotes new bone formation in sinus augmentation procedures. To the best of our knowledge, this is the first human report in oral surgery applications generally, and in sinus augmentation in particular, to demonstrate the predictability and efficacy of this biomaterial. Thus, these HA/TCP-augmented sites accommodated osseointegrated implants either at the simultaneous or the staged surgical approaches.

Clinically and histologically, no difference was encountered, either in simultaneous or delayed implant placement at 6 and 9 months post-augmentation as well as regarding the residual bone height. As this study examined the augmentation material in the surrounding area of the implants and not the bone-implant contact, differences between one or two-stage approaches were not anticipated.

Although no difference was encountered in grafted particle resorption between 6 and 9 months, an increase in newly formed bone was evident. Bone primarily surrounded the grafted particles and was further affected by their osteoconductivity. The high density of cellular osseous tissue close to the particle margins and the further increase of the bone area fraction, as observed between the two examined periods, verify this assumption. Thus, the present human data confirms previous animal studies (Hashimoto-Uoshima et al. 1995;

Table 1. Percentage area fractions of newly formed bone and grafted HA/TCP particles

| Specimen | Gender/age | Type of surgery | Time of harvesting (months) | Bone | Particles |
|----------|------------|-----------------|-----------------------------|------|-----------|
| 1 | M/55 | One-stage | 6 | 29.5 | 10.9 |
| 2 | F/45 | One-stage | 6 | 32.9 | 7.8 |
| 3 | M/65 | One-stage | 9 | 26.3 | 36.1 |
| 4 | F/52 | One-stage | 6 | 15.1 | 57.2 |
| 5 | F/75 | Two-stage | 6 | 36.4 | 26.3 |
| 6 | M/52 | One-stage | 9 | 42.3 | 40.1 |
| 7 | M/50 | Two-stage | 6 | 25.1 | 22.1 |
| 8 | F/45 | One-stage | 6 | 26.4 | 22.8 |
| 9 | F/75 | Two-stage | 9 | 41.2 | 24 |
| 10 | M/65 | One-stage | 9 | 32.2 | 23.7 |
| 11 | M/50 | Two-stage | 6 | 24.8 | 35.3 |
| 12 | F/45 | One-stage | 6 | 38.7 | 31.7 |
| 13 | M/65 | One-stage | 9 | 40.4 | 16.9 |
| 14 | F/37 | Two-stage | 9 | 53.8 | 18.4 |
| 15 | F/65 | Two-stage | 6 | 27.8 | 23.4 |
| 16 | F/50 | One-stage | 6 | 17.6 | 28.2 |
| 17 | F/54 | Two-stage | 6 | 35.6 | 18.5 |
| 18 | F/37 | Two-stage | 9 | 40 | 16.3 |
| 19 | M/60 | One-stage | 6 | 18.1 | 24.8 |
| 20 | M/51 | One-stage | 6 | 34.1 | 23.4 |
| 21 | F/31 | One-stage | 9 | 48.1 | 34.2 |
| 22 | F/31 | One-stage | 9 | 51.5 | 27.5 |
| 23 | M/63 | Two-stage | 9 | 40.5 | 29 |
| 24 | F/37 | Two-stage | 9 | 31.7 | 13.7 |
| 25 | F/65 | Two-stage | 9 | 40.7 | 12.8 |
| 26 | M/63 | Two-stage | 6 | 37.8 | 24.5 |
| 27 | F/54 | Two-stage | 9 | 52.1 | 12.4 |
| Average | | | | 34.8 | 24.5 |

HA, hydroxyapatite; TCP, tricalcium phosphate; M, male; F, female.

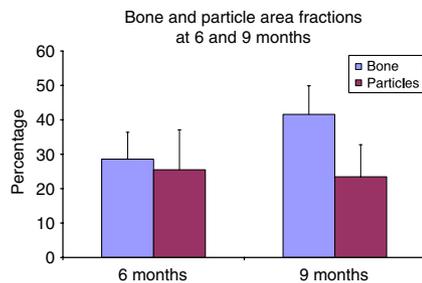


Fig. 5. Bone and particle area fractions at 6 and 9 months.

Mankani et al. 2001; Le Nihouannen et al. 2005), and other biomaterials (Hallman et al. 2001; Artzi et al. 2004).

In most sites examined histologically, the grafted particles were surrounded by

cellular osseous tissue in direct contact. Some areas of bone tissue displayed lamellar structure, a possible sign of remodeling of the initial bone formed.

Clinically, upon specimen harvesting, residual layers of the overlying collagen membrane were occasionally identified. The presence of an underlying osseous layer, just under the membrane has also been reported when non-resorbable membranes were examined histologically (Ohnishi et al. 2000). Close histologic observation has also shown that a calcification process might occur in these remnants (Tal et al. 2008; Zubery et al. 2007).

In the present study, the average area fraction of newly formed bone area fraction

resembled that found in other studies with different biomaterials (Artzi et al. 2001, 2003, 2005; Hallman et al. 2001). In an experimental calvarial defect in rabbits, the average area fraction of newly formed bone was 20% at 4 weeks (Jung et al. 2006).

Although HA/TCP is considered to be a fully resorbable material, our observations reveal that particle area fraction was similar to that of other biomaterials, i.e., alloplasts and xenografts, which have been evaluated (Artzi et al. 2001, 2003, 2005). The morphometric data disclosed only slight resorption of the examined biomaterial between 6 and 9 months, which was non-significant. A longitudinal controlled trial as conducted on bovine bone mineral and β -TCP biomaterials (Artzi et al. 2004) should be considered to determine the resorption rate of this material.

Bone growth between 6 and 9 months occurred in between the particles without significant particle removal. The fact that bone and particle area fractions did not exhibit significant correlations suggests that the two tissue elements changed independently.

Conclusion

The interconnected biphasic HA/TCP is an osteoconductive and biocompatible material. As a bone graft material mixed with cortical autogenous bone chips, it promotes newly formed bone in sinus augmentation procedures which increases over time.

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