CLINICAL ORAL IMPLANTS RESEARCH

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# Maxillary sinus augmentation with microstructured tricalcium phosphate ceramic in sheep

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**Key words:** biomaterials, bone substitutes, tricalcium phosphate, maxillary sinus augmentation

**Abstract**

**Objective:** The objective of this study was to evaluate the biological performance of osteoinductive microstructured tricalcium phosphate (MSTCP) particles in maxillary sinus floor augmentation surgery in sheep.

**Material and methods:** Sinus floor augmentation was performed in eight Swifter sheep. In each animal, the maxillary sinus floor was unilaterally augmented with MSTCP particles. Computed tomography (CT) imaging and histological analyses were performed after 12 weeks of implantation.

**Results:** Maxillofacial CT, histology, histomorphometrical analysis and sequential polychrome fluorescent labeling indicated that MSTCP particles provided a scaffold for cell ingrowth and bone formation. After a 12-week implantation period, the sinuses grafted with MSTCP showed an increased bone height of 6 mm and a mean total bone volume of 43%, with significant degradation of MSTCP particles.

**Conclusion:** MSTCP particles represent a suitable bone substitute material for maxillary sinus floor augmentation surgery.

Placement of dental implants requires the presence of adequate alveolar bone quantity and quality. In case the amount of alveolar bone or its quality is insufficient, additional surgical techniques are needed to achieve primary implant stability (Kaufman 2003). For extensive alveolar defects, onlay or inlay grafting procedures have been advised (Boyne & James 1980; Misch 1987; Tong et al. 1998; Kaufman 2003; Esposito et al. 2006). To allow implant placement in the posterior part of the maxilla, sinus floor augmentation surgery has become a routine procedure (Merkx et al. 2003; Wallace & Froum 2003; Del Fabbro et al. 2004; Emmerich et al. 2005) that results in an implant survival rate of over 90% for 3–5 years (Hurzeler et al. 1996; Nkenke & Stelzle 2009).

Autologous bone grafts are considered the gold standard in sinus floor augmentation (Browaeys et al. 2007; Klijn et al. 2010a). However, harvesting an autologous bone graft, especially from extra-oral sources, is associated with several disadvantages. Especially, reservations must be made regarding the prolonged operating time and donor site morbidity (Sindet-Pedersen & Enemark 1990; Cohen et al. 1991; Hoppenreijs et al. 1992; Eppley 2005), which may include hypersensitivity (Damien & Parsons 1991), pelvic instability, infection (Canady et al. 1993; Swan & Goodacre 2006) and paraesthesia (Beirne et al. 1996). Consequently, various allogenic, xenogenic and synthetic graft materials or combinations thereof have been used as an alternative to autologous bone grafts, with variable clinical results (Nkenke & Stelzle 2009; Klijn et al. 2010a, 2010b).

Meta-analysis of augmented maxillary sinuses demonstrated comparable newly formed bone volumes for different types of biocompatible, osteoconductive bone substitutes, mostly calcium phosphate (CaP) ceramics (Klijn et al. 2010a). In an advanced setup, such synthetic CaP-based bone substitutes have been combined with autologous bone, growth factors or even as a fully tissue engineered cellular construct (Browaeys et al. 2007; Klijn et al. 2010a) to establish osteoinductive capacity. These composites combine the advantages of each element alone, i.e. osteoconductive properties from the synthetic material and osteoinductive capacity from biological components. Nevertheless, the disadvantage of harvesting bone or the expenses of using growth factors are still present using this approach. Furthermore, the efficacy of cell-based constructs remains unclear as evidenced by conflicting experimental results reported using various cell types of animal or human origin (Meijer et al. 2007; Park 2010).

**Date:**
Accepted 17 February 2011

**To cite this article:**
Klijn RJ, Hoekstra JWM, Van Den Beucken JJJP, Meijer GJ, Jansen JA. Maxillary sinus augmentation with microstructured tricalcium phosphate ceramic in sheep. *Clin. Oral Impl. Res.* **xx**, 2011; 000–000.
doi: 10.1111/j.1600-0501.2011.02190.x

In view of safety, regulatory and application issues, the ideal synthetic bone substitute should be available off the shelf and have intrinsic osteoinductive capacity. Winter and Simpson (1969) were the first to report ectopic bone formation induced by a biomaterial. Since then, several authors reported material-induced osteogenesis in soft tissues in different animal models (Yuan et al. 2001a, 2001b). A recent study of Yuan et al. (2008, 2010) showed an excellent osteoinductive capacity of microstructured tricalcium phosphate (MSTCP) particles after 12 and 52 weeks of intramuscular implantation in dogs and sheep. Consequently, it was hypothesized that MSCTP particles represent a suitable bone substitute in maxillary sinus floor augmentation surgery.

In view of this, the present study aimed to evaluate the biological performance of MSTCP particles (Yuan et al. 2008) with osteoinductive capacity in maxillary sinus floor augmentation procedures in sheep.

## Material and methods

### Material

MSTCP particles were kindly provided by RevisiOs BV (Bilthoven, the Netherlands). The production of the particles was described before (Yuan et al. 2008). In brief, CaP powders (with a Ca/P ratio 1.5) were mixed with an $H_2O_2$ solution and naphthalene particles to produce slurries. After foaming, drying and evaporation of the naphthalene, the material was sintered for 8 h at 1100°C. X-ray diffraction analysis of MSTCP showed more than 90% β-tricalcium phosphate (β-TCP) phase and a trace of hydroxyapatite (<10 wt%) (Yuan et al. 2008). After milling, ceramic particles with a size of 150–500 μm were sieved, cleaned and sterilized using γ-irradiation (Isotron Nederland BV, Ede, the Netherlands).

### Animals

In total, eight female Swifter sheep in a healthy condition (average weight: 65 kg) were used in this study. The experimental protocol was reviewed and approved by the Experimental Animal Ethical Committee of the Radboud University Medical Center, the Netherlands (RU DEC 2008-194). National guidelines for the care and use of laboratory animals were observed.

### Surgical procedure

To reduce the perioperative infection risk, prophylactic antibiotics were administered subcutaneously (Albipen® 15%, 3 ml/50 kg preoperative and Albipen® LA, 7.5 ml/50 kg for 3 days postoperative, Intervet BV, Boxmeer, the Netherlands). General anesthesia was initiated by an intravenous injection of pentobarbital (AUV Wholesale, Cuijk, the Netherlands). Subsequently, the sheep were intubated and connected to an inhalation ventilator with a constant volume of a mixture of nitrous oxide, isoflurane and oxygen. The animals were immobilized in a ventral position and the operation site was shaved, washed and disinfected with povidone-iodine. Access to the maxillary sinus was obtained by first exposing the facial antral wall over a 4-cm-long paramedian sagittal skin incision, taking care to avoid the facial artery. After reflecting the skin flap, a bony window with a diameter of around 10 mm was created rostrally with a dental burr (Elcomed® 100, W&H Dentalwerk Burmoos GmbH, Bürmoos, Austria) under continuous external cooling, followed by careful removal of the resultant bone plate from the Schneiderian membrane. The membrane was then elevated from the buccal and caudal bony wall and displaced cranially with bent blunt dissectors. In total, eight maxillary sinuses were unilaterally grafted with 2 ml of the MSTCP particles. The grafted defect was covered by the bone plate. Thereafter, the soft tissues were closed in separate layers. To reduce pain after surgery, all sheep received Finadyne® (AUV Wholesale) for 3 days postoperatively. Directly after sinus augmentation surgery and after 6 weeks of healing time, a radiograph was obtained of the maxillary sinus area to verify graft localization and surgery.

### Sequential fluorescent labeling

A polychrome sequential fluorescent labeling method was carried out in seven sheep to visualize the dynamics of bone growth; one sheep was used as a control to exclude auto fluorescence of the specimens and implanted MSTCP after histological processing. Fluorescent labels oxytetracycline (blue), alizarin complexon (red), calcein (green) and tetracycline (blue) were administered subcutaneously (25 mg/kg body weight) at 1, 3, 6 and 9 weeks post surgery, respectively.

### Sample retrieval and histological processing

Animals were sacrificed using an overdose of Pentobarbital 12 weeks post surgery. The maxilla with surrounding tissue was retrieved. Subsequently, the sinus region was excised and excess tissue was removed. Using a diamond saw, the tissues were sawed into smaller blocks suitable for histological processing. Specimens were fixed in a phosphate-buffered formaldehyde solution (pH 7.4), dehydrated in a graded series of ethanol (70–100%) and finally embedded in polymethymetacrylate (PMMA). Multiple histological sections ($n \geq 3$; ~ 20 μm) were prepared in a buccal–palatinal direction at consecutive levels through the grafted area using a microtome with diamond blade (Leica Microsystems SP 1600, Nussloch, Germany). Histological sections were stained with methylene blue and basic fuchsin. Two unstained sections (~ 20 μm) were prepared for fluorescent microscopy of each block. Furthermore, MSTCP granules were embedded in PMMA, after which sections (~ 20 μm) were prepared of the as-received granules before implantation.

### Radiological evaluation

After sacrifice, cone beam-computed tomograms (CBCT) (i-CAT™ 3-D Imaging System, Imaging Sciences International Inc., Hatfield, PA, USA) were made of the maxillae, during which grafts were localized and the maximum augmentation height was measured at three levels using I-Cat Vision® software (Imaging Sciences International Inc.).

### Histological and histomorphometrical analysis

The histological evaluation consisted of a morphological description of at least three sections of each grafted area using a light microscope (Leica Microsystems AG, Wetzlar, Germany). Fluorescent labeling was observed with unstained sections using a fluorescent microscope (Leica Microsystems AG). Excitation wavelengths for each of the fluorescent labels were as follows: 365–490 nm/520–570 nm (blue), 530–580 nm/600–645 nm (red) and 436–495 nm/517–540 nm (green). The fluorescence images were merged with one regular image using transmission light microscopy to assess the dynamics of bone formation over time.

In addition, the stained sections were scored quantitatively using computer-based image analysis techniques (Leica Qwin Pro-image analysis system). Three randomly selected standardized areas (1.4 $mm^2$) within the boundaries of the grafted area of at least three histological sections were analysed of each specimen (Figs 1 and 2b).

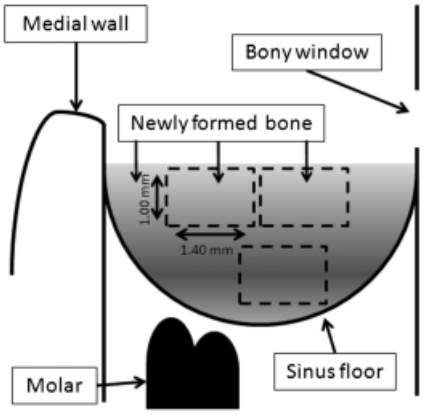


*Fig. 1.* Schematic overview of three randomly selected standardized areas (1.4 $mm^2$) within the boundaries of the grafted area for histomorphometric analysis.

The area of newly formed bone, residual MSTCP and connective tissue were determined and expressed as a percentage within this region of interest by manual selection based on pixel value detection. Furthermore, the perimeter of the granules in direct contact with bone was measured and expressed as a percentage of the total perimeter for ~ 500 randomly chosen particles from different histological sections. Additionally, the surface, perimeter and longest axis of these granules in the histological sections were compared with sections of the granules before implantation.

#### Statistical analysis

Quantitative measurements were expressed as median and mean ± standard deviation (mean ± SD). The differences in the particle surface, perimeter and longest axis were analysed using the Mann–Whitney test. Statistical analysis was performed using SPSS statistical software package (IBM® SPSS 16.0). Data were considered significant at $P<0.05$.

## Results

#### General observations of the animals

The surgical procedure was uneventful for all animals. At sacrifice, a total of eight maxillary sinuses including surrounding tissue could be retrieved. Macroscopically, no signs of infection or adverse tissue reaction were observed.

#### Radiological evaluation

Directly after sinus augmentation and after 6 weeks of healing time, an X-ray was obtained of the maxillary sinus area. The location of the surgical site and graft location were examined by comparison. No dislocations or adverse effects were observed (data not shown). A mean augmented height of 6 ± 2.2 mm was found in CBCT scans after 12 weeks of implantation time.

#### Histology

No signs of inflammation or adverse tissue reactions were observed. Bony structures were preserved and the maxillary sinus did not change in shape. Newly formed woven bone was observed bridging the space between the original bone of the buccal wall and the sinus floor (Fig. 2a). This bone appeared as vital bone tissue containing osteoblasts, osteoid covering the border and osteocytes inside bone lacunae. Furthermore, bone marrow-like tissue was observed in between the bone voids, including blood vessels. The Schneiderian membrane was completely covering the augmented sinus floor (Fig. 2a). The remaining MSTCP granules could be easily identified in the newly formed bone by its size, shape and dark color (Fig. 2b). The bone was in close contact with the surface of MSTCP granules without the presence of an intervening fibrous tissue layer. In areas where the remaining MSTCP particles were in contact with bone marrow, occasionally, multinucleated cells were observed at the surface of the particles, suggesting the occurrence of cell-mediated resorption (Fig. 2c). Overall, MSTCP particles appeared to be reduced in size compared with their original size.

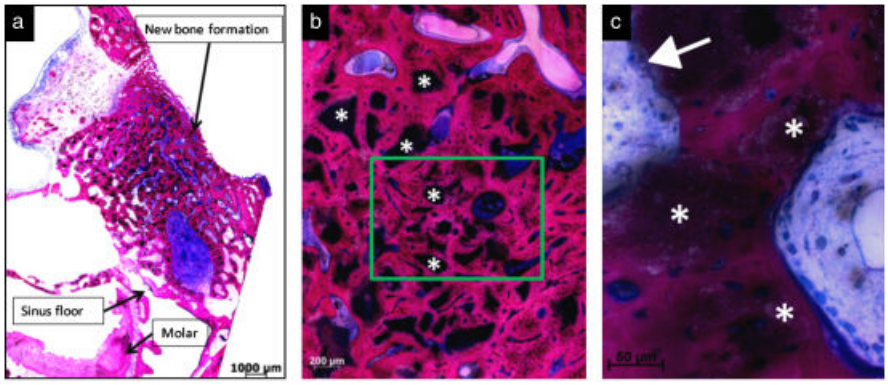


*Fig. 2.* Histology 12 weeks after maxillary sinus augmentation in sheep Histological overview of the sinus area (a). Higher magnification of the microstructured tricalcium phosphate (MSTCP) particles embedded in bone and direct bone bonding (b); occasionally, multinucleated cells were observed in contact with MSTCP particles (c). Haematoxylin–eosin staining. Green square specify an example of a randomly selected area (1.4 mm²) within the boundaries of the grafted area for histomorphometry; *MSTCP particle; arrow indicates a multinucleated cell.

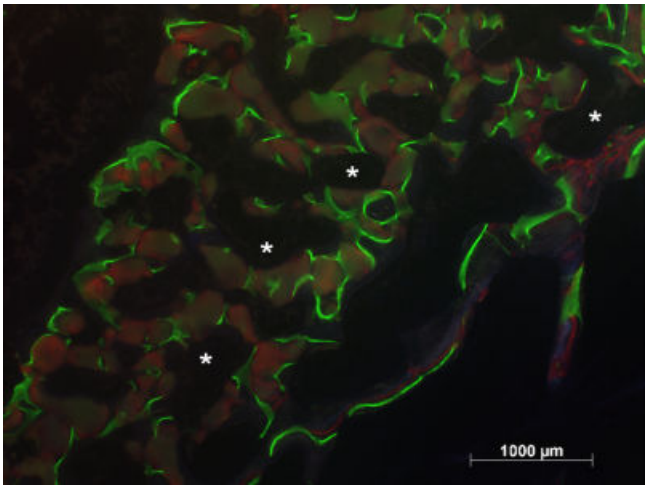


*Fig. 3.* A polychrome sequential fluorescent labeling Fluorescent labels oxytetracycline (blue), alizarin complexon (red), calcein (green) and tetracycline (blue) after 12 weeks of implantation time of the microstructured tricalcium phosphate (MSTCP) group (*MSTCP particle).

#### Fluorochrome labeling

Fluorescent labels were systemically administered to allow the visualization of dynamic bone formation at 1, 3, 6 and 9 weeks. All sequential fluorochrome markers could be identified at a low magnification within the total grafted area in the experimental site (Fig. 3). Consecutive fluorochrome markers' appearance, laid down in the form of bands, was observed around the MSTCP granules. No clear sequence of fluorescent bands could be observed within the grafted area. In contrast to the labels tetracycline (blue; administered in week 1 and 9) and calcein (green; administered in week 6), the presence of the alizarin complexon label (red; administered in week 3) could not be identified as sharply in the sections.

#### Histomorphometry

##### Tissue formation

Figure 4 demonstrates the results of the histomorphometric analysis of the MSTCP-augmented specimens. The area fraction of the newly formed bone ranged from 25% to 65.4%. Randomly selected areas of the histological sections revealed a mean newly formed bone area of 42.9 ± 9.7% (median: 42.3%). Furthermore, a mean area of 33.1 ± 10.9% (median: 23.8%) was occupied by fibrous connective tissue.

##### Particle degradation

Particle surface, perimeter and longest axis were measured before and after implantation to assess particle degradation (Fig. 5 and Table 1).

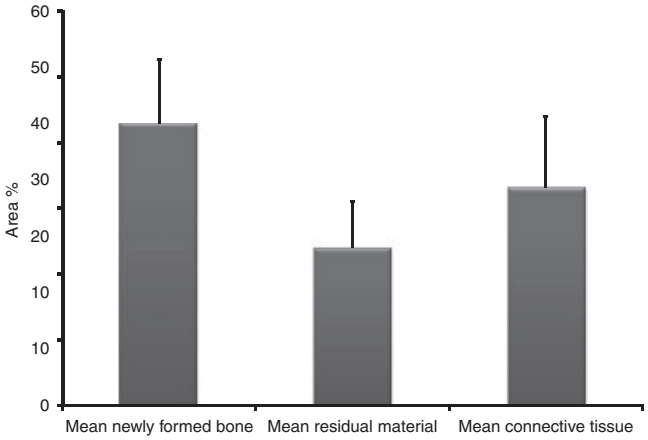


*Fig. 4*. Histomorphometry mean percentage of newly formed bone, residual material and connective tissue area in the microstructured tricalcium phosphate (MSTCP) group.

A significant ($P<0.001$) decrease in all the parameters was measured between the starting material and the MSTCP particles 12 weeks after implantation (Table 1). The mean residual particle area recorded for MSTCP was 24 ± 7% (median: 34%) after 12 weeks (Fig. 4). Furthermore, the percentage of bone in contact with the graft particles was measured to determine the actual bone to particle contact percentage. A mean bone particle contact of 82.3 ± 7.5% (median: 81.7%) was found (Table 1).

## Discussion

This pre-clinical study aimed to evaluate the biological performance of MSTCP particles applied in maxillary sinus augmentation surgery in sheep with an implantation period of 12 weeks. Histology, histomorphometric analysis, sequential fluorescent labeling and maxillofacial CBCT were used to systematically evaluate new bone formation and bone remodeling in the grafted area. Substantial new bone formation and incorporation of the MSTCP particles into the newly formed bone were observed. These observations indicate a successful performance of MSTCP particles. Additionally, MSTCP particles showed signs of degradation.

Experimental studies on maxillary sinus floor augmentation have been reported using different animal species and various grafting materials. The sheep model is considered one of the suitable larger animal models for maxillary sinus augmentation surgery, due to its similarity in size, bone physiology and structure to the human maxillary sinus (Estaca et al. 2008). It was, however, not possible to perform the procedure using an intra-oral approach like in humans, due to the limited opening of the mouth of a sheep. Consequently, this model only allows sinus augmentation using an extra-oral approach, as already several authors stated before (Haas et al. 1998a, 1998b; Jakse et al. 2003; Brumund et al. 2004). In contrast to the finding that Schneiderian membrane perforation is a common technical problem in humans (Karabuda et al. 2006), the thickness of the sheep Schneiderian membrane showed enough consistency to perform sinus floor augmentation without tearing the membrane in any case.

Autologous bone grafting is considered the gold standard in sinus floor augmentation surgery (Klijn et al. 2010a). However, a variety of alloplastic bone substitutes, single or in combination with autologous bone, have been used in sinus augmentation surgery in humans, with various results (Nkenke & Stelzle 2009; Klijn et al. 2010a). TCP was already used in the 1970s to heal bone defects (Cameron et al. 1977). Nkenke & Stelzle (2009) analysed the present literature to determine whether there are advantages of using autologous bone over bone substitutes in sinus floor augmentation procedures with respect to dental implant survival. They concluded that no evidence exists that either supports or refutes the superiority of autologous bone grafts over TCP with regard to dental implant survival (Nkenke & Stelzle 2009). Furthermore, a recent meta-analysis by Klijn et al. (2010a) demonstrated that histomorphometrically determined bone volumes did not differ significantly from using an autologous bone graft or using TCP in maxillary sinus augmentation. Also, the addition of autologous bone to TCP appeared to have a negligible effect regarding total new bone formation (Klijn et al. 2010a). According to these findings, the MSTCP particles that were used in this preclinical study showed a substantial amount of new bone formation.

The ultimate bone substitute in implant dentistry should eventually be resorbed and replaced by functional newly formed bone. Therefore, the use of resorbable TCP particles for sinus floor elevation has received increasing attention in implant dentistry (Szabo et al. 2005; Knabe et al. 2008). Resorption of MSTCP particles was demonstrated both histologically and histomorphometrically 1 year after intramuscular implantation in dogs (Yuan et al. 2008). In the present study, resorption could be confirmed by comparing particle size, perimeter and longest axis before and after implantation in the bony maxillary sinus environment. Yuan et al. (2008) demonstrated both chemical dissolution and cell-mediated resorption over time. Considering the occasional presence of multi-nucleated cells in contact with remaining MSTCP particles, the present study corroborates these findings.

In the past few years, several authors described the use of osteoconductive β-TCP in human maxillary sinus floor augmentation procedures (Szabo et al. 2001, 2005; Zerbo et al. 2001, 2004; Ozyuvaci et al. 2003; Zijderveld et al. 2005; Suba et al. 2006; Simunek et al. 2008). Histomorphometrically obtained bone volumes of 17–52% were found in patients after 6–12 months of healing time. Besides, the use of TCP has been evaluated in maxillary sinus augmentation in various animal species. In a recent study of Wang et al. (2010), TCP was used as a grafting material for maxillary sinus augmentation in dogs. A mean newly formed bone area of 34% was found after 24 weeks of healing time. In another study of Jiang et al. (2009), TCP was used in sinus augmentation in rabbits; after 8 weeks of healing time, a mean newly formed bone area of 16% was found. TCP was also used in a miniature pig study, as described by Gruber et al. (2008), who installed, in contrast to the current study, a dental implant simultaneously with sinus floor augmentation. A mean bone volume up to 19% was found after 12 weeks (Gruber et al. 2008). The MSTCP particles evaluated in this study in sheep resulted in 43% of newly formed bone after 12 weeks after implantation within the grafted area.

Zerbo et al. (2004) concluded that due to the absence of osteoinductive properties of the TCP they investigated, the rate of bone formation was delayed in comparison with autologous bone grafting. It would be beneficial for the patient to reduce the interval between maxillary sinus augmentation and implant placement by accelerating the process of integration of the grafted material. Some authors state that the application of osteoinductive substances, such as platelet-rich plasma (PRP) or growth factors, is a promising option (Marx et al. 1998; Park 2009). However, PRP seemed not to be beneficial for new bone formation in sinus augmentation (Plachokova et al. 2008) and the use of recombinant growth factors is an expensive option. With the

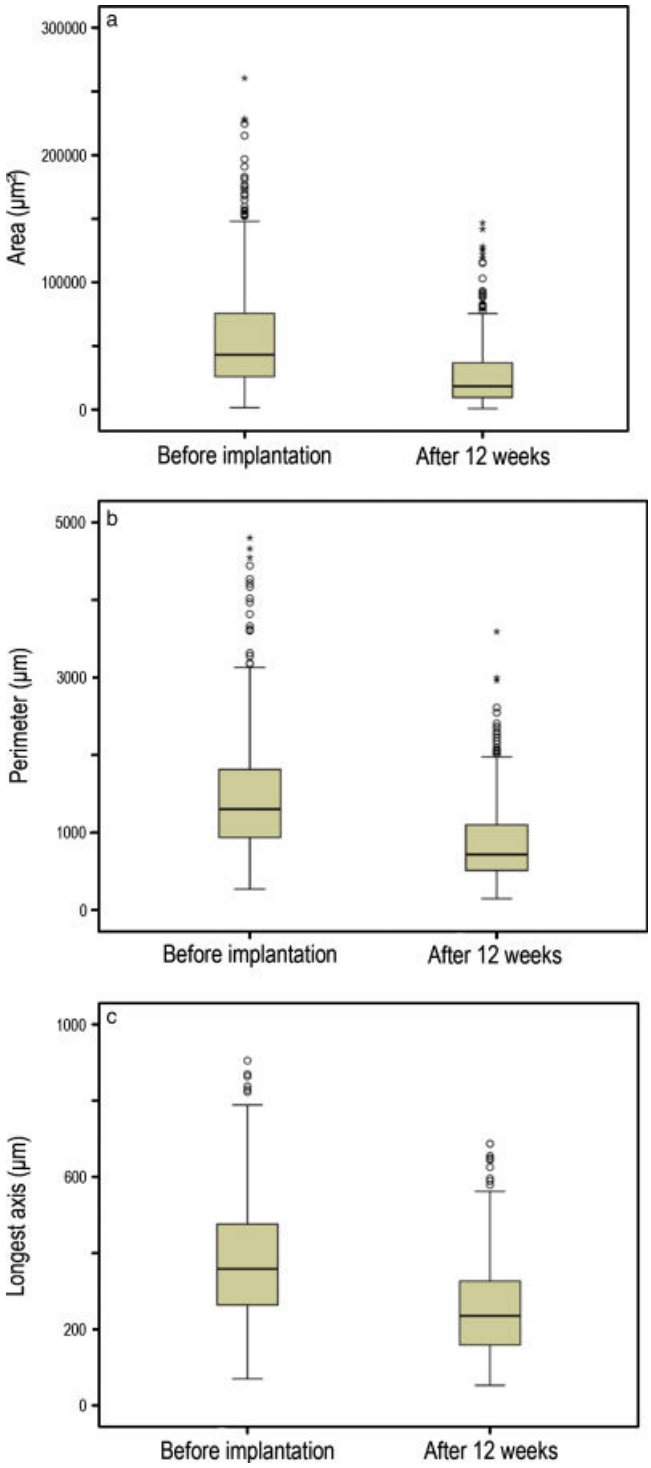


*Fig. 5*. Box plot of microstructured tricalcium phosphate (MSTCP) particle degradation MSTCP particle characteristics before and after 12 weeks of implantation time in maxillary sinuses of sheep. (a) MSTCP particle area (median: 43,153 µm²). (b) MSTCP particle perimeter (median: 1300 µm). (c) MSTCP particle longest axis (median: 358 µm).

***Table 1*. MSTCP particle degradation MSTCP particle characteristics before and after a 12-week implantation time in maxillary sinuses of sheep**

| $N \approx 500$ | Area (µm²) | Perimeter (µm) | Longest axis (µm) | Bone particle contact (%) |
|---|---|---|---|---|
| $T = 0$ | 58,718 ± 47,043 | 1487 ± 803 | 386 ± 159 | – |
| $T = 12$ weeks | 26,927 ± 25,273 | 851 ± 490 | 258 ± 126 | 82.3 ± 7.5 |
| Mann–Whitney | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ | |

MSTCP, microstructured tricalcium phosphate.

ability to form bone in soft tissue (Yuan et al. 2008), the osteoinductive MSTCP particles used in this study will be useful to speed up the process of appositional bone growth. Sequential fluorochrome markers were observed within the total grafted area implicating bone growth through the hole specimen already at least 3 weeks after implantation. However, the question remains what healing period is necessary to provide adequate bone formation for successful dental implant placement. Furthermore, MSTCP particles were not evaluated at an ectopic site in this study. Therefore, no conclusions regarding the osteoinductive properties could be drawn. Follow-up studies with prolonged evaluation periods and the application of a combined approach with both sinus floor augmentation and one- or two-staged implant placement will provide an insight into bone to dental implant contact and mechanical stability of the augmented bone.

## Conclusion

Based on the histological and histomorphometrical results of this preclinical study, MSTCP particles were shown to represent a suitable bone substitute material for maxillary sinus augmentation. The MSTCP demonstrated to provide a scaffold for cell ingrowth and substantial bone formation. Additionally, MSTCP particles showed significant signs of degradation after 12 weeks of implantation in the sheep maxillary sinus.

**Acknowledgements:** The authors acknowledge RevisiOs BV (Bilthoven, the Netherlands) for providing the MSTCP particles and Dr E. M. Bronkhorst for statistical help. The authors gratefully acknowledge the support of the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science.

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