

Injectable bone substitute based on β -TCP granules

Results from an *in-vivo* analysis in Wistar rats

Authors M. Barbeck, J. Lorenz, C. Landes, R.A. Sader, C.J. Kirkpatrick & S. Ghanaati, Germany

Introduction

In the recent years biomaterial research has focused on developing a reliable and safe alternative to autologous bone for augmentation in case of a reduced local bone amount. As autologous bone has osteoinductive, osteoconductive and osteogenic properties, it is postulated to be the gold standard in peri-implant hard tissue augmentation.¹ Xenogenic bone substitutes, originating from animals of different species and processed in different steps, are well researched and accepted from both surgeons and patients.² Alloplastic bone substitutes from synthetically manufactured hydroxyapatite (HA), beta-tricalcium phosphate (β -TCP) or a mixture of these two compounds have been reported to be biocompatible, degradable and osteoconductive.³⁻⁷

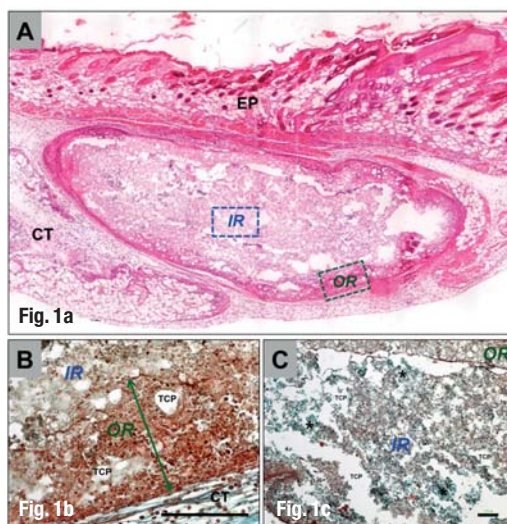
During integration in the host tissue, parameters such as the potential induction of an inflammatory response, the biomaterial vascularisation and degradation play an important role.⁸⁻¹⁴ By modifying the chemical and physical characteristics of a biomaterial, i.e. its chemical composition and its surface structure morphology and porosity, it seems to be possible to tailor alloplastic bone substitute materials individually to specific requirements.⁹ From a number of *in vitro* and *in vivo* trials it is known that beside the chemical composition, the granule size also has a significant impact in the degradation behaviour of synthetic bone substitute materials. Granules with a mean size larger than 500 μm and a low porosity are more slowly degraded and resist the ingrowth of connective tissue in the implantation bed more than granules smaller than 50 μm .^{9,12,15-17} However, small granules might be more suitable for different kinds of defect classes.

With a combination of small, pure-phase β -TCP granules, which serve as bioactive fillers, and a carrier matrix of methylcellulose (MC) and hyaluronic acid (HY), the fast degradation of the small granules and the connective tissue influx might be prevented by the aqueous phase. All three components, the β -TCP, the MC and the HY are known to be biocompatible^{2-6,18-25} and have optimal mechanical and regulating properties, which are favourable for tissue engineering and regeneration.²⁰⁻³⁵ Additionally, biomaterial might also be easier to handle, as it is paste-like and therefore injectable into the augmentation site.

The aim of the present study was to investigate the inflammatory response, as well as the overall integrity within the implantation bed, the degradation behav-

Fig. 1 The tissue reaction to the triphasic bone substitute material at day 3 after implantation:

- a) a total scan of the bone substitute, which was located within the connective tissue (CT: connective tissue; EP: Epidermis; OR: outer cell-rich region; IR: inner region) (H&E-staining, total scan, 100x magnification);
- b) the infiltration of mononuclear cells into the outer region (Movat's pentachrome staining, 400x magnification; scale bar = 100 μm);
- c) inner region (IR), in which the β -TCP granules (TCP) were embedded (asterisks) (Movat's pentachrome staining, 100x magnification; scale bar = 100 μm).





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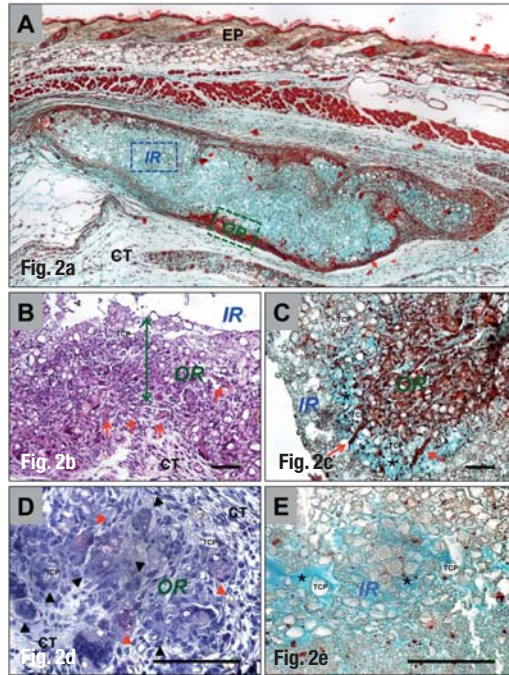
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Fig. 2_ The tissue reaction to the triphasic bone-substitute material at day 10 after implantation:

- a) an overview of the total implant area by means of a total scan. (H&E-staining, total scan, 100x magnification);
- b) the outer region (OR, double head arrow), which was distinguishable from the inner region (IR). (red arrows: vessels; arrow heads: giant cells; H&E-staining, 200x magnification; scale bar = 100 μ m);
- c) ingrowth of connective tissue into the outer region of the implanted paste-like bone-substitute material. (red arrows: vessels; red asterisks: polymer solution; Movat's pentachrome staining, 400x magnification; scale bar = 100 μ m);
- d) multinucleated giant cells within the outer region; red arrow heads: TRAP positive multinucleated giant cells; black arrow heads: giant cells without TRAP activity (TRAP-staining, 400x magnification; scale bar = 100 μ m);
- e) inner region of the implantation bed. (arrows: mononuclear cells; asterisks:) aqueous polymer solution (Movat's pentachrome staining, 200x magnification; scale bar = 100 μ m).



our and the vascularisation of a new paste-like bone-substitute material composed of β -TCP, methylcellulose (MC) and hyaluronic acid (HY) by means of histological and histomorphometrical analysis. Therefore, the cellular tissue reaction to this new bone substitute material was investigated in the subcutaneous implantation model in Wistar rats during an observation period of 60 days. Three groups with (a) subcutaneous implantation of pure, solid β -TCP, (b) subcutaneous injection of sodium chloride, and (c) a sham operated animals served as controls.

Material and methods

Bone substitute material

In the present study an injectable bone substitute paste made from crushed pure-phase β -TCP, methylcellulose and hyaluronic acid was investigated. The manufacturing process of sintering and crushing results in ceramic particles with a size of $<63 \mu$ m, which were mixed with an aqueous polymer solution in a ratio of 70 wt% ceramics and 30 wt% polymer solution.

Study design

With approval from the Committee on the Use of Live Animals in Teaching and Research of the State of Rhineland-Palatinate, Germany, 90 female, 5-week-old Wistar rats were divided in three groups and received implantation of the above mentioned injectable β -tricalcium phosphate bone substitute material (group 1) and pure, solid β -TCP granules (group 2). For control, ten animals underwent an operation with an injection of sodium chloride (group 3) and another 20 animals (group 4) underwent preparation of subcutaneous pockets without biomaterial implantation. At 3, 10, 15, 30 and 60 days, the animals were sac-

rificed by an overdose of ketamine and xylazine and according to a previously described method^{9, 36-39} the bone substitute material was explanted and processed for histological and histomorphometrical analysis.

Tissue preparation

The extracted samples were fixed in 4% formalin, cut into segments of 4 mm thickness, decalcified, dehydrated in alcohol and embedded in paraffin. Afterwards the samples were cut with a microtome in sections of a thickness of 4 μ m and stained as follows: the first section was stained with haematoxylin and eosin (H&E), the second section with tartrate-resistant acid phosphatase (TRAP) to identify osteoclast-like cells, while the third and fourth section were used for immunochemical staining with ED-1 antibody (for cells of the monocyte-macrophage lineage). A fifth slide was stained with Movat's pentachrome to visualise connective tissue ingrowth within the implantation bed and a seventh slide was stained by von Kossa/Safranin-O staining for identification of calcium and calcium phosphates.^{9, 36-41}

Histological and histomorphometrical investigation

After staining, the sections were investigated by independent investigators with a diagnostic microscope (Nikon, Tokyo, Japan) and the tissue-biomaterial interaction within the implantation bed and the peri-implant tissue was examined histomorphometrically using the NIS-Elements software (Nikon, Tokyo, Japan). The total number of vessels and their area on each slide were determined and related to the total implantation area. Thereby, for each time point, a mean number of vessels per square millimetre and a mean total vessel area could be determined. The results of the quantitative analysis were presented as mean \pm standard deviation with differences considered significant if p-values were <0.05 (* $p < 0.05$) and highly significant if p-values were <0.01 (** $p < 0.01$).

Results

All the animals in each group survived the surgical procedures and the postoperative observation period without complications. No signs for severe inflammatory response were observed.

Tissue reaction to β -TCP granules

Beginning on day 3, the β -TCP granule group material induced penetration of phagocytes, macrophages and connective tissue fibres, resulting in a poorly vascularised fiber and fibroblast rich granulation tissue, which had completely penetrated the implantation bed at day 15. At day 30 and 60 only few remnants of the bone substitute granules were obvious. The vascularisation of the implantation bed remained low, presenting no significant differences in

the aforementioned vascularisation parameters to the results of the two control groups (Figs. 7a-d, 8a & b):

Tissue reaction to paste-like β -TCP solution

Within the implantation bed of the triphasic paste-like β -TCP at day 3, the bone-substitute material appeared as a compact structure. The implanted material could be divided in compact outer surface and an inner core. A large number of phagocytes, lymphocytes, a few plasma cells and eosinophils and connective tissue fibres started to penetrate the outer surface without reaching the inner core. Therefore neither vessels nor connective tissue or organic structures were found in the central parts of the implantation bed (Figs. 1a-c).

At day 10 the separation within the biomaterial was still present. The outer structure contained an active granulation tissue, with an increased vascularisation by newly formed vessels, while the inner core, comparable to day 3, was still populated by very few mononuclear cells (Figs. 2a-e).

At day 15, the degradation of the outer structure proceeded. The granulation tissue formed around the biomaterial was rich in vessels and contained more multinucleated giant cells than at day 10. In the inner core still less connective tissue fibres and mononuclear and especially multinuclear cells were detectable compared to the outer regions (Figs. 3a-d).

The implantation bed showed total integration of the inner and outer part of the implanted biomaterial at day 30. TRAP positive multinucleated giant cells dominated the fiber and vessel rich granulation tissue. The former outer region had been transformed into a connective tissue with very few phagocytes and rich in fibres, while the inner core of the implant had been transformed into a richly vascularized granulation tissue (Figs. 4a & c).

To the end of the observation at day 60 the degradation of the biomaterial, mainly by multinucleated giant cells continued. In areas, where biomaterial remnants were still present, granulation tissue was still present, while in parts where the biomaterial was already completely degraded, it was replaced by adipose and connective tissue. Remaining granules were surrounded by phagocytes, i.e. macrophages and multinucleated giant cells (Figs. 5a & c, 6a-d).

Histomorphometric results

Histomorphometric investigation of the explanted biomaterials was performed to determine the vascularisation within the implantation bed at different time points of biomaterial integration. At day 3 a mild vascularisation within the three phasic injectable

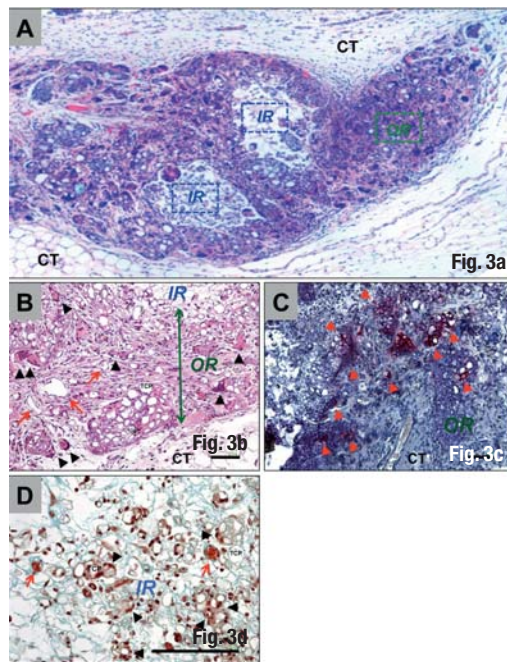


Fig. 3 The tissue reaction to the triphasic bone-substitute material at day 15 after implantation; a) overview of the implanted material within the subcutaneous connective tissue (CT). (H&E-staining, total scan, 100x magnification); b) outer region (OR, double head arrow), with a unique granulation tissue (red arrows: vessels; arrow heads: multinucleated giant cells; (H&E-staining, 200x magnification; scale bar = 100 μ m); c) TRAP activity in multinucleated giant cells (red arrow heads; TRAP staining, 200x magnification; scale bar = 100 μ m); d) the composition of the inner region (IR) of the implanted material. (arrow heads: multinucleated giant cells; red arrows: vessels; black arrows: mononuclear cells (Movat's pentachrome staining, 400x magnification; scale bar = 100 μ m).

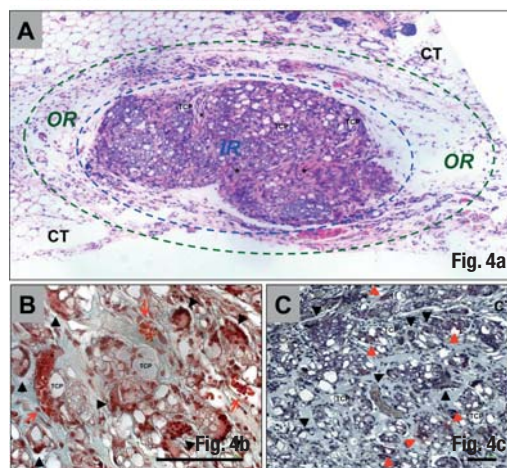


Fig. 4 The tissue reaction to the triphasic bone-substitute material at day 30 after implantation; a) total scan of the implant region. Inner circle with few bone substitute remnants (asterisks: granulation tissue; H&E staining, total scan, 100x magnification); b) the former inner region transformed into a granulation tissue. (red arrows: vessels; arrow heads: multinucleated giant cells; Movat's pentachrome staining, 400x magnification; scale bar = 100 μ m); c) differential TRAP expression at this time point shown by TRAP-negative and TRAP-positive multinucleated giant cells (black arrow heads: TRAP- negative multinucleated giant cells; red arrow heads: TRAP- positive multinucleated giant cells; TRAP-staining, 200x magnification; scale bar = 100 μ m).

β -TCP biomaterial, which was significantly higher than in the β -TCP granule group, was observed (** $p < 0.01$, Figs. 8a & b). At days 10, 15, 30 and 60 significantly higher values for percent vascularisation and vessel density in the β -TCP paste were observed compared to the solid β -TCP and the two control groups (sham operation and sodium chloride). These data indicated a maturing of the vessels within the implant. A detailed overview of the significance levels between the different groups at each time point is given in figure 8a & b.

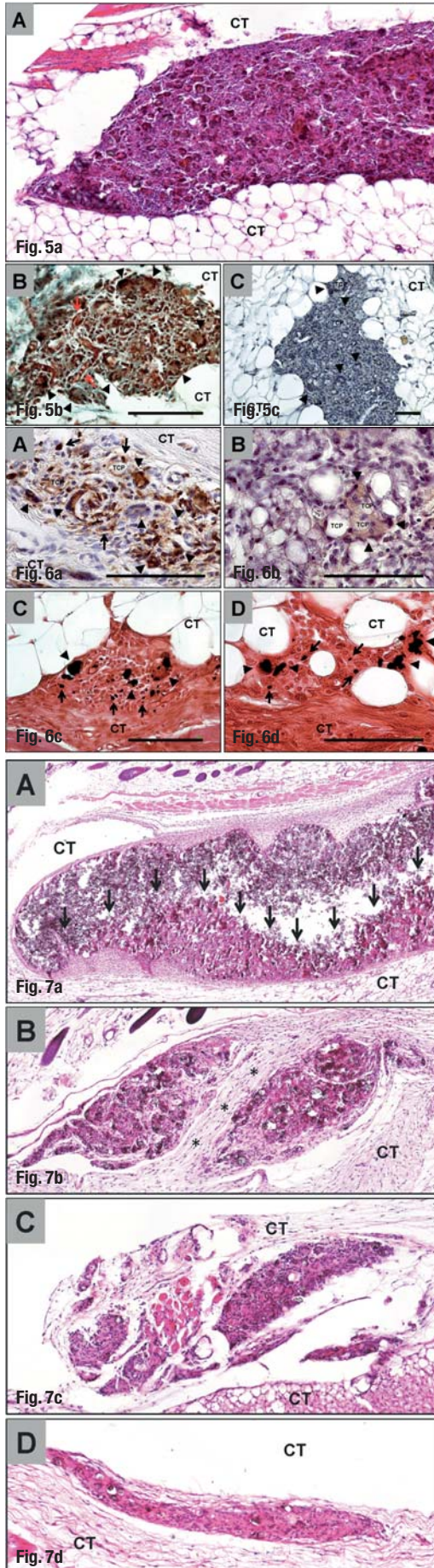
_Discussion

In the present study the tissue reactions to a paste-like bone substitute material composed of β -TCP, methylcellulose and hyaluronic acid was investigated in the subcutaneous implantation model in Wistar rats over 60 days. Implantation of pure solid β -TCP, injection of sodium chloride and sham operation served as controls. The primary focus of histological and his-

Fig. 5 The tissue reaction to the triphasic bone-substitute material at day 60 after implantation; a) total scan of the implant area (H&E staining, total scan, 100x magnification); b) the remaining granulation tissue, i.e., vessels (red arrows), macrophages and multinucleated giant cells (arrow heads); (Movat's pentachrome staining, 400x magnification; scale bar = 100 µm); c) only TRAP-negative giant cells (arrow heads) were detectable at this time point (CT = connective tissue, TCP = β-TCP granules) (TRAP staining, 200x magnification; scale bar = 100 µm).

Fig. 6 The immuno- and histochemical analysis of the cellular degradation of the triphasic bone-substitute material; a) overview of the implantation bed at day 60. (arrows: macrophages; arrow heads: multinucleated giant cells; ED-1 staining, 400x magnification); b) higher magnification showing a single multinucleated giant cell (arrow heads; ED-1 staining, 600x magnification; scale bar = 100 µm); c and d) The implantation bed at day 60, in which mononucleated (arrows) and multinucleated giant cells (arrow heads) were involved in the cellular degradation of the β-TCP granules (black granules) (Von Kossa/Safranin-O staining); C: 200x magnification; D: 600x magnification; scale bar = 100 µm).

Fig. 7 The tissue reaction to the β-TCP granules used in the control group; a) implantation bed at day 10. (arrows: tissue ingrowth covering approximately half the area of the implantation bed; H&E staining, total scan, 100x magnification); b) implantation bed at day 15. (asterisks: granulation tissue divided by bridges of connective tissue; H&E staining, total scan, 100x magnification); c) implantation bed at day 30. (asterisks: fragmentation within single islands by connective tissue bridges; H&E staining, total scan, 100x magnification); d) implantation bed at day 60. Almost complete degradation of the β-TCP granules (H&E staining, total scan, 100x magnification).



tomorphometrical analysis was on the characterisation of the cellular response to the bone-substitute material, its stability and the vascularisation of the implantation bed.

The histological analysis showed the triphasic biomaterial remaining in a bulk-like structure with an inner core and an outer ring for up to 30 days. A wall of vessel-rich granulation tissue was formed at the tissue-biomaterial interface on the outer wall, which the initial degradation seemed to originate from. The aqueous solution seemed to hold the bone substitute material in the inner core and prevent it from early ingrowth of connective tissue. At the end of the study period the inner region becomes invaded by more degrading cells, which penetrated towards the β-TCP granules. β-TCP is a well-established bone substitute material which is highly biocompatible, cellular degradability and supports osseointegration and osteoconduction.³⁻⁶ Therefore, the analysis of the pure solid β-TCP granules showed early invading of the biomaterial granules by mono- and multinucleated phagocytes, i.e., macrophages and giant cells from the peri-implant tissue. It is known, that these cells are expressed in the foreign body response and take part in the biomaterial degradation process.⁸

The histomorphometric analysis of the extracted and processed samples revealed significantly higher vascularisation within the paste-like triphasic β-TCP group compared to the pure β-TCP group. This increased vascularisation started in the outer core and was initiated by multinucleated giant cells within the implantation bed.^{8,9}

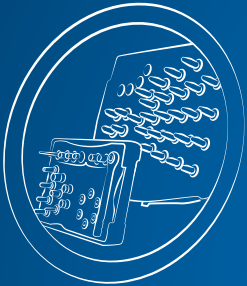
By physicochemical changes in material characteristics, such as size, porosity and shape, synthetic bone substitute can be individually tailored to achieve an optimal level of inflammation and vascularisation in order to regulate bone tissue regeneration.^{9,39}

Origin of the bone substitute material as well as production and processing parameters, such as sintering temperature, play an important role in material stability. Hydroxyapatite (HA) is known to be more stable than β-TCP.³⁻⁵ Fast degradation results in the risk of connective tissue ingrowth in the implantation bed of β-TCP augmentation material, which might compromise osteoconduction. Another approach to enlarge material stability is the here presented combination of β-TCP, Methylcellulose and Hyaluronic acid, which leads to a divided inner and outer structure of the biomaterial and inhibits the connective tissue ingrowth between the granules in the inner core. The concept of a paste-like material combines not only the advantages of different material classes, but also simplifies the augmentation process by a minimally-invasive insertion. Cellulose, used in this study as aque-



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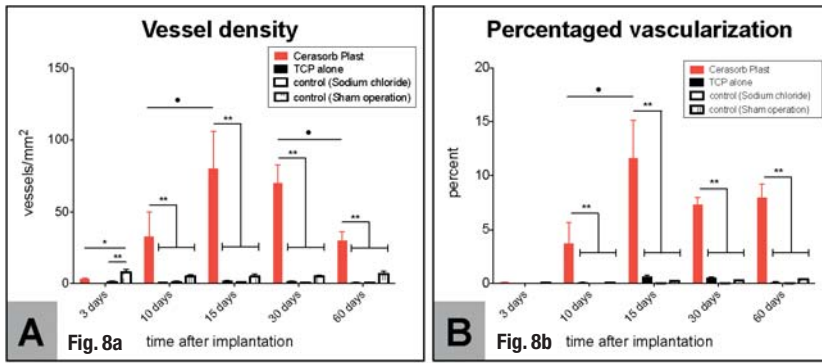


Fig. 8 Histomorphometrical analysis of the vascularization of the implantation beds of the triphasic paste-like bone-substitute material group and the three control groups, i.e., animals with β -TCP granules, sham-operated animals, and animals injected with saline: a) vessel density and (b) percentage vascularisation (*/** = interindividual statistical significances, •/•• = intraindividual statistical significances).

ous matrix for the β -TCP granules, is a well-known and -researched polymer from wood fibres and part of vegetable cell walls.^{18,19} In orthopaedic surgery, the hydroexpansivity of cellulose is used for cellulose-based implants to delay the resorption of bone substitute defects.^{18-22,42,43}

Hyaluronic acid, the third component of the investigated injectable bone substitute material, is a linear polymer of repeating disaccharide units composed of D-glucuronic acid and N-acetyl-D-glucosamine and can be found in many tissues of the human body, such as skin, cartilage and the vitreous humour, and is well-suited to applications in tissue regeneration.^{23-25,31-35}

In different studies investigating HY, the binding capacity, the ability to communicate with cell surface receptors and to modulate the inflammatory response, contributing to the stabilisation of the granulation tissue matrix within the implantation bed were shown.⁴⁴⁻⁴⁶ In tissue engineering the mechanical stability, osteoconductivity and the ability to promote the migration and differentiation of osteoblasts make HY suitable for bone tissue augmentation.^{23,25,47,48} From the histological and histomorphometrical results it can be concluded, that both cellulose and HY contribute in a synergistic way to the hydroexpansivity, to the mechanical stability of the implant and to control the connective tissue ingrowth towards the central part of the biomaterial. Another advantage of the triphasic bone substitute material presented in this study is its injectability. In combination with its hydroexpansivity, all parts of the defect can be reached by the biomaterial minimal-invasively. Further, the lifetime and the stability of the biomaterial is enhanced and the connective tissue ingrowth is decelerated.

In previous trials paste-like bone-substitute materials with other compositions and qualities have shown promising results and positive influence on bone regeneration.⁴⁹⁻⁵¹ The results of the present study support the assumption that injectable bone-substitute materials with the above-mentioned components can be used as osteoconductive filling mate-

rials for reliable and successful bone tissue augmentation.

Conclusion

In the present study, the tissue reaction to a triphasic paste-like bone substitute material of β -TCP, cellulose and hyaluronic acid was investigated in the subcutaneous implantation model in Wistar rats over a time period of 60 days. Implantation of pure solid β -TCP, injection of sodium chloride and sham operation served as controls. By histological and histomorphometrical methods, the cellular reaction, the inflammatory response and the vascularisation within the implantation bed were analysed.

The combination of β -TCP, Cellulose and Hyaluronic acid was shown to generate a two phasic bulk with an inner core of β -TCP granules and an outer core of an aqueous solution, which inhibited the premature ingrowth of connective tissue in the intergranular space within the first 30 days. Further, and in contrast to the control groups, the outer structure induced the formation of multinucleated giant cells, which resulted in a higher vascularisation of its implantation bed.

Concluded, the combination of small β -TCP granules, cellulose and hyaluronic acid is a new, promising concept for a bone substitute material that can be easily applied via minimally invasive surgical techniques.

Acknowledgements

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Dr Dr Shahram Ghanaati

Department for Oral, Cranio-Maxillofacial and Facial Plastic Surgery, Medical Center of the Goethe University Frankfurt, Frankfurt/Main, Germany

shahram.ghanaati@kgu.de

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