Biocompatibility of β-Tricalcium Phosphate Root Replicas in Porcine Tooth Extraction Sockets – A Correlative Histological, Ultrastructural, and X-ray Microanalytical Pilot Study

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ABSTRACT: This investigation studies porcine tissue response in tooth extraction sockets treated with root replicas made out of β-tricalcium phosphate (β-TCP; β-Ca₃(PO₄)₂) granules, molded and held together by thermal fusion of

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a thin film of polyglycolic–polylactic acid copolymer. Six left mandibular third incisors \( (n = 6) \) of experimental pigs are treated with the root replicas and four contralateral incisors are used as nontreated controls \( (n = 4) \). Two animals each were killed at 20, 40, and 60 weeks of observation periods. The mandibular jaw segments were prepared in toto for light microscopy by resin embedding and serial ground sectioning. Additionally, one \( \beta \)-TCP-treated socket at 60 weeks was thoroughly investigated by correlative light, electron microscopic and electron probe X-ray microanalysis to assess the bioabsorbability and host removal of the replica material from the implant site. The extraction wounds of the animals healed satisfactorily with very little histologically observable differences in the healing pattern of the test and control sites. The \( \beta \)-TCP was completely removed from extracellular sites, but at 60 weeks, remnants of it were found in the cytoplasm of multinucleated giant cells. The root replicas made out of \( \beta \)-TCP were biocompatible and bioabsorbable. Osseous healing occurred both in the test and control sockets, but the healing process was delayed due to the presence of \( \beta \)-TCP particles.

**KEY WORDS:** alveolar process atrophy, alveolar ridge preservation, biocompatibility, oral implants, oral wound healing, osseointegrated dental prostheses, tooth extraction sockets.

**INTRODUCTION**

*The long-term prognostic, functional, and aesthetic success of the conventional noninvasive and osseointegrated dental prostheses depends on the presence of sufficient volume of healthy jaw bone [1]. The latter relates to proper postextraction healing and rehabilitation of the alveolar process. The morphological events of human alveolar socket healing after tooth extraction have been well documented [2]. It has long been recognized that healing of tooth extraction sockets is associated with a catabolic remodelling of the residual alveolar process that results in loss of large volume of the jaw bone [3]. This atrophy of the alveolar process has been described as a ‘...chronic progressive, irreversible and disabling disease...’ [4] or reduction of residual ridges (RRR, [5]). The etiology of the condition is unknown. Nevertheless, both systemic and local factors have been suggested to be involved [5,6]. The rate of RRR is rapid immediately after tooth extraction and the process is particularly pronounced in the anterior maxilla.*

Various preoperative and intraoperative measures have been attempted to preserve the alveolar process. Careful oral hygiene and precautions that minimize trauma during tooth extraction procedures limit postoperative inflammation and associated bone loss. Other measures include: (a) retention of the natural roots so as to preserve the alveolar process, (b) insertion of prefabricated semianalogous root form implants, and (c) application of various forms of guided bone
regeneration (GBR) techniques. Alloplastic bone substitutes [7–17] and bone or osseous derivatives of isogenous [1,18–20], allogenous [7,11, 21,22], and xenogenous [23–25] origin have been used as osseoinductive and/or osteoconductive materials in various GBR techniques. Numerous attempts have been aimed at maintaining [7,9–15,18,23–29] or regenerating (or augmenting) [1,8,16,17,19–22, 30,31] the alveolar process. Most of the publications were human case reports [7,10,14,16,18,20, 22,23,25,28,29,32] or animal studies [9,12,17,19,21, 26,30] with varying and often conflicting outcomes assessed by clinical and radiographic criteria. In several cases, there was histological examination of the tissues removed during implant surgery [1,7,10,11,16–19,21,23–25,30].

Biodegradable osteosynthesis devices have been successfully used for internal fixation of fractures in maxillofacial and orthopedic surgeries [33,34]. The idea has been advanced [35] that custom-made biodegradable root replicas applied as immediate implants in extraction sockets could preserve the alveolar process. The animal experimental application of polyglycolic acid (PGA) and subsequent limited human therapeutic usage of polylactic acid (PLA) root replicas have been reported to preserve the alveolar ridge [35,36]. Copolymers of polyglycolic–polylactic acids (PLGA) eventually replaced the homopolymers for such clinical applications [15,37].

Clinical reports on bioabsorbable synthetic polymers, albeit limited, have been positive, but investigations on PLGA showed an in vitro drop of pH of a phosphate buffered (PBS) medium to well below 3 as a result of degradation [38] of the copolymers into acidic monomers. In the body, the latter are expected to be metabolized yielding energy, CO₂, and water via the citric acid cycle [15]. However, an in vivo lowering of pH around the implanted root replicas can happen as a result of imbalance in the release and elimination of the acidic degradation products with possible adverse effects, such as demineralization of the residual bone [37], inflammation, and even necrosis of tissues surrounding the replica.

Research [38] has shown that granules of β-tricalcium phosphate (β-TCP, β-Ca₃(PO₄)₂) can be held together by thermal fusion of a very thin film of PLGA coating of the granules. The resulting agglomeration of β-TCP granules can be shaped to any form by using appropriate molds. On melting, the thin PLGA film would glue the β-TCP granules together exposing the surface of the latter, but would not lower the pH of the surroundings on degradation. The near spherical shape of the β-TCP granules ensures an interstitial porosity of the resultant product open to the exterior. Therefore, the β-TCP with low amounts of PLGA as a binding agent has been suggested [38] to provide an almost neutral degradable
material to treat tooth extraction sockets for the prevention of alveolar bone loss.

It is mandatory to study the reaction of mammalian tissues to chemicals and foreign objects that are intended for various implant therapies before such materials are clinically applied on a large scale in human and animal patients. Therefore, the objectives of this investigation were: (a) to study the histological response to treating tooth extraction sockets with freshly prepared root replicas of $\beta$-TCP in a miniature swine model and (b) to answer the question as to whether or not the $\beta$-TCP is completely removed from the site of implantation.

**MATERIALS AND METHODS**

**Animals and Teeth**

A total of 10 teeth from six healthy adult miniature swines (Table 1) were involved in this study. The pigs were kept under appropriate farm conditions with food and water ad libitum. Two animals were randomly allotted to each of the three observation groups, namely, 20, 40, and 60 weeks, respectively. Physical examination of the animals, photo-, radiographic recordings and subsequent invasive oral surgical procedures were done under general anesthesia at the scheduled times, in accordance with German legislation on protection of animals, veterinary professional standards and support. In addition to general anesthesia, infiltration local anesthesia was used during tooth extraction to minimize bleeding and postoperative pain. In all six animals, the extraction site of the left mandibular third incisor (tooth 33) was treated with a root replica that was freshly prepared as described below. In four

<table>
<thead>
<tr>
<th>Animal</th>
<th>Observation (weeks)</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Test socket</th>
<th>Control socket</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRP-1</td>
<td>20</td>
<td>6</td>
<td>M*</td>
<td>33</td>
<td>43</td>
</tr>
<tr>
<td>SRP-2</td>
<td>20</td>
<td>6</td>
<td>F</td>
<td>33</td>
<td>–</td>
</tr>
<tr>
<td>SRP-3</td>
<td>40</td>
<td>3</td>
<td>F</td>
<td>33</td>
<td>43</td>
</tr>
<tr>
<td>SRP-4</td>
<td>40</td>
<td>3</td>
<td>F</td>
<td>33</td>
<td>43</td>
</tr>
<tr>
<td>SRP-5</td>
<td>60</td>
<td>3</td>
<td>F</td>
<td>33</td>
<td>43</td>
</tr>
<tr>
<td>SRP-6</td>
<td>60</td>
<td>6</td>
<td>F</td>
<td>33</td>
<td>–</td>
</tr>
</tbody>
</table>

M* = castrated male; F = female. Tooth 33 is mandibular left third incisor; tooth 43 is mandibular right third incisor.
of the six animals, the contralateral extraction site of tooth 43, was not treated with the root replica as a split mouth control. The animals were not used for any other experimental purpose. It is noteworthy that third mandibular incisors are the smallest of porcine teeth that are amenable to extraction without extensive trauma to the teeth and the surrounding bones. Our own attempt to extract a second mandibular incisor in one animal resulted in only a fractured crown of the tooth. Therefore, apart from ethical considerations, the number of test and control sockets could not be increased by making use of more extraction sockets in the experimental animals involved.

**Preparation of the Root Replicas**

After extraction, the six test teeth were rinsed briefly in running cold water and the soft tissue attached to the root surface was removed mechanically. The replicas were prepared ‘chairside’ with β-TCP granules coated with a thin layer of PLGA (PLGA 50:50, Boehringer Ingelheim, Ingelheim, Germany) as described in detail elsewhere [38]. Briefly, the granules (500–800 μm) were poured into the freshly prepared root impression mold of the extracted tooth. The mold containing the granules was then heated to a temperature of 80°C. The PLGA surface film of the granules melt at 70°C so that the granules glue together to form a root-shaped body on cooling. The replicas revealed an interconnected porosity of about 50% (v/v) with an average pore diameter of 200 μm. Preparation of the root replica molds took about 5 min.

**Clinical Procedures**

After careful extraction of the teeth to avoid damaging the alveolar processes of the animals, root replicas were inserted into the alveoli after a few minutes. The edges of the gingival wounds were held together by sutures that also retained the replicas in the alveoli. The sutures were removed after 10 days of application. Empty contralateral alveolar sockets, sutured after tooth extraction, served as controls. The animals were clinically examined, photographed, and radiographed at various intervals during the observation periods. After appropriate period of observations, the animals were killed under general anesthesia at the determined times (Table 1). Thereafter, the lower jaws were dissected out, briefly rinsed in cold water and immersed in large volumes of half-strength Karnovsky’s fixative [39]. The fixative solution was changed
after 24 h and the specimens were further fixed in cold storage (4°C) for 4–6 weeks.

**Tissue Processing**

The lower jaws of the six animals were divided along the midline into left and right halves using a diamond disc (Vari/Cut, Leo Corporation, St. Joseph, USA). Each half was then trimmed to remove the mandibular portion distal to the canines. Using a band saw (Exakt, Norderstedt, Germany) the mesial segments of the mandibles so prepared were further subdivided mesiodistally through the midplane of the extraction sockets of teeth 33 and 43, respectively. In the case of one animal of 60 weeks of observation period, a central slice of about 0.8 mm thickness was removed from the test side and saved for Epon® (Fluka AG, Buchs, Switzerland) embedding. The buccal and lingual segments of each mandibular specimen were dehydrated in ascending grades of ethanol, thoroughly infiltrated with and thereafter embedded in the commercial resin Technovit® 7200 VLC (Kulzer, Wehrheim, Germany). The 0.8-mm thick central lower jaw segment removed from the test side of the animal with 60 weeks of observation period was substantially trimmed to reduce the specimen size to the area of the healing extraction socket of the tooth 33 that could be clearly orientated in a stereomicroscope. The non-demineralized specimen was postfixed in 1.33% osmium tetroxide (OsO₄) buffered in 0.067 M s-collidine, dehydrated in ascending grades of ethanol and embedded in Epon®.

**Scanning Electron Microscopy (SEM)**

The cutting faces of the polymerized Technovit® blocks were polished with silicon carbide grinding paper followed by a polishing cloth with diamond paste of 3, 1, and 0.25 grain size. The polished block faces were then coated with a carbon layer of about 10–15 nm thickness using a MED020/EVM030 electron beam evaporator (BAL-TEC, Balzers, Lichtenstein) and examined in the backscatter mode in a Stereoscan 180 scanning electron microscope (SEM, Cambridge, Dortmund, Germany) equipped with a four-quadrant backscatter detector setup to show atomic number contrast. Digital electron micrographs were prepared at 15–20 kV of accelerating voltage and a working distance of about 20 mm using a personal computer connected to the SEM and the software WinDISS (Point Electronic, Halle, Germany).
Ground Sections and Light Microscopy

Mesiodistal serial sections of about 50 μm thickness were prepared using the microgrinding system [40] (Exakt, Norderstedt, Germany). The sections were stained in toluidine blue and photomicrographed using a photomicroscope M420 (Leica, Glattbrugg, Switzerland) equipped with a Coolpix® 4500 (Nikon Corporation, Tokyo, Japan) digital camera.

Transmission Electron Microscopy (TEM) and Energy Dispersive Analysis of X-rays (EDAX)

From the Epon® block, 1–1.5 μm thick survey sections were prepared using a histodiamond knife (Diatome AG, Biel, Switzerland) and the Reichert Ultracut E microtome (Leica, Glattbrugg, Switzerland). The sections were stained in periodic acid-Schiff (PAS) and methylene blue-Azur II and photomicrographed in bright and polarized lights using a Dialux 20 photomicroscope (Leica, Glattbrugg, Switzerland) equipped with the digital camera Progress C14 (Jenoptik, Eching, Germany) and an electronic imaging system (ImageAccess, Imagic, Glattbrugg, Switzerland). After locating an ideal site for ultrastructural evaluation and analysis, the selected area in the epon block was target trimmed and thin sectioned with the Reichert Ultracut E microtome (Leica, Glattbrugg, Switzerland). Some of the thin sections were double contrasted with lead and uranium salts [41,42] and examined in a Philips EM400T transmission electron microscope (Philips, Endoven, The Netherlands). Noncontrasted thin sections were used for EDAX with the aid of a Philips CM120 scanning transmission electron microscope (STEM, Philips, Endoven, The Netherlands).

RESULTS

Healing of the extraction wounds of the test and control sites in all the animals was satisfactory by qualitative visual and radiographic assessments at regular intervals. No signs of microbial infection, exudation, or dehiscence of the wound could be observed at the time of suture removal. Macroscopically, the gingival dimensions and bony contours maintained the respective preoperative appearances as could be judged by standardized photographic and radiographic means.
General Histology

Treatment of the porcine tooth extraction sockets with $\beta$-TCP (Figure 1(A)–(C)) did not result in clear histomorphologically observable advantages in the osseous healing pattern (Figure 1(A)–(C)) in comparison to untreated contralateral control sites (Figure 1(D)–(F)). At 20 weeks postoperatively, a distinct extraction socket was visible in both the test (Figure 1(A)) and control (Figure 1(D)) specimens. Spicules of bone could be observed to project into the extraction sockets from the bottom and lateral walls of the alveoli. Horizontal extension of the alveolar ridge seemed to narrow the cervical opening of the extraction socket. At 40 weeks (Figure 1(B) and (E)) the sockets were partially traversed by trabecular bone that formed a loose osseous network. The latter became closer fitting at 60 weeks (Figure 1(C) and (F)) of observation. A complete closure of the cervical opening of the alveolar ridge could be observed in the test sites at 40 and 60 weeks (Figure 1(B) and (C)), compared to interruptions noticeable in ridge healing of the contralateral controls (Figure 1(E) and (F)).

SEM Visualization

Backscattered imaging in SEM at 20 weeks revealed distinct extraction sockets at the test (Figure 2(A)) and control sites. The former contained a substantial amount of electron-dense fine particles distributed throughout the socket. At 60 weeks (Figure 2(B)), a distinct trabecular network of bone could be observed around the socket area that was much smaller than that in the 20-week specimens. The 60-week specimens (Figure 2(B) and (C)) contained similar electron-dense particles as those observed at 20 weeks, but in much lesser quantity. At a higher magnification (Figure 2(C)), the jaw bone displayed in two distinct areas of electron densities. While most of the bone was of high electron density, an osseous layer of varying thickness, but of distinctly lesser electron density, lined the healing and remodeling front of the socket wall.

Polarization Microscopy and TEM Visualization

The cellular and subcellular details of the test socket at 60 weeks were analyzed using various microtechniques in Epon® embedded semithin (1.5 $\mu$m) and thin (80 nm) sections. The healing socket consisted of
Figure 1. Overview photomicrographs showing the histological status of healing porcine extraction sockets (ES) of tooth 33 in animals at 20 (A), 40 (B), and 60 (C) weeks postextraction that were implanted with biodegradable root replicas. Contralateral extraction sockets of tooth (43), not treated with root replicas, provided the controls (D–F). There were no histologically observable differences in the osseous healing of the extraction sockets, except for the complete closure (B, C) of the alveolar ridge in the test cases as against the interruptions noticeable in the ridge healing of contralateral controls (arrows in E, F). 32, 42 = left and right mandibular second incisors, respectively. Original magnifications: (A)–(F), ×4.8.
trabecular bone delimiting large pockets of well-vascularized soft connective tissue that contained plenty of fat globules (Figure 3(A)).

Except for the presence of occasional lymphocytes, the area was free of infiltrating inflammatory cells, such as polymorphonuclear leucocytes.

**Figure 2.** Low magnification backscattered scanning electron micrographs (A, B) of the surfaces of two synthetic resin embedded specimens. Note the extraction sockets (ES) of teeth 33 at 20 (A) and 60 (B) weeks of observation periods, respectively. Figure 2(A) originates from that shown histologically in Figure 1(A). The rectangular demarcated area in (B) is magnified in (C). Note the highly electron dense particles at 20 weeks post-extraction (arrow in A). Much less material is still visible in the 60-week postextraction specimen (arrows in B, C). The repairing osseous socket wall has a lower electron density (arrowheads) than the rest of the bone. Original magnifications: BO = bone. (A) ×17.5, (B) ×15, (C) ×62.
(PMN) and plasma cells. But a large number of multinucleated giant cells, in isolated clusters, could be observed (Figure 3(B) and (C)). Their nuclei were distributed randomly throughout the cytoplasm. The cytoplasm revealed inclusion bodies that appeared empty in bright field (Figure 3(C)) but intensely birefringent in polarized light (Figure 3(D)) so that the area of the section appeared to be studded with brightly illuminating particles against a dark background. Electron microscopically (Figure 4(A)), the inclusion bodies contained a highly electron-dense material that remained intact within the inclusion bodies when
Figure 4. A transmission electron micrograph (A) showing profiles of giant cell cytoplasm (GC) and nuclei (NU). Note the highly electron-dense material in numerous inclusion bodies (IB) in the cytoplasm. X-ray microanalysis (B) of the electron-dense material at the intersection of the two hairlines in the inset (arrow) revealed the presence of predominantly calcium and phosphorus elements at the site. The same analysis done at the intranuclear (C, arrow in the nucleus) and intact cytoplasmic control sites did not reveal the presence of elemental calcium and phosphorus. Original magnification: (A) ×4400.
these were delimited by plasma membranes. However, when pushed away from the original sites during sectioning, the electron-dense material showed signs of disintegration. The birefringent electron-dense bodies were observed only within the giant cells and not in the interstitial extracellular areas.

**X-ray Microanalysis**

The giant cell inclusion bodies (Figure 4(B)), several cytoplasmic and nuclear sites (Figure 4(C)) were subjected to element analysis using a STEM/EDAX unit. The dye-resistant, birefringent, and electron-dense inclusion bodies consistently revealed peaks of energy range of elemental calcium (Ca) and phosphorus (P). Only one significant peak of X-ray emission, in the range of carbon (C), could be registered from the cytoplasmic and nuclear sites.

**DISCUSSION**

This study presents correlative histological, ultrastructural, and X-ray microanalytical data on the biocompatibility and host removal of β-TCP-made root replicas applied as an immediate implant after tooth extraction in a miniature swine model.

The idea [35] of applying biodegradable root replicas as immediate implants in extraction sockets to preserve the alveolar process has not been adequately tested by independent animal and human clinical trials. Only two publications [35,36] describe the treatment of extraction sockets with biodegradable root replicas. In an experimental study in rabbits [35], it was found that insertion of custom-made root replicas made out of PGA into the extraction sockets of second maxillary incisors prevented palatal collapse of the implanted area. Contralateral control sockets that did not receive any root replicas showed clear collapse of the area. Subsequently, in a woman patient [36], one extraction socket was treated with a chairside-made PLA root replica. Only radiographic follow-up was possible and it was reported that ridge height could be preserved during an observation period of 84 weeks. In a larger clinical trial [37] with eight human patients, solid and porous forms of PLGA copolymers were used as root replicas before insertion of definite metallic implants. In some of the cases, an initial decline in the radiodensity of the alveolar processes surrounding the extraction sockets could be observed, which suggested initial bone demineralization.
The first aim of this study was to visualize potential tissue reaction and healing pattern of porcine tooth extraction sockets treated with β-TCP root replicas at predetermined observation periods. Serial ground sections of anterior mandibular regions of the experimental pigs containing treated and contralateral nontreated sites at 20, 40, and 60 weeks showed gradual bone healing of the extraction sockets. No clearly observable advantages in the healing pattern of the extraction sockets could be found between the test and control sites by the conventional bright field microscopy. The absence of complications, such as tissue necrosis or signs of inflammation indicates biocompatibility of the root replicas. No quantitative assessment of the tissue components of the healing alveolar sockets was done, since the small sample size is unlikely to yield any more meaningful biological data of appropriate statistical reliability.

The second objective was to investigate whether or not the β-TCP was completely removed from the site of implantation during a clinically relevant period of observation. As cellular details and the fine particles of β-TCP could not be adequately resolved in 50-μm histological ground sections, other suitable microanalytical methods were used to determine the degree of β-TCP removal. Visualization of the treated and nontreated control sockets in backscattered mode of SEM clearly showed the presence of fine electron-dense particles in test sites, the amount of which declined with increasing observation periods. Low amounts of electron-dense material were still present in the specimens of the longest observation period. However, backscatter images could not clarify whether the particles were intra- or extracellular in nature.

The presence of clusters of multinucleated giant cells in the soft tissue of the healing socket at 60 weeks indicated a foreign body host response. The physical properties of the inclusion bodies, such as dye resistance, birefringence, and high electron density are suggestive of the presence of inorganic element(s). Electron probe microanalysis consistently confirmed the presence of calcium (Ca) and phosphorus (P). The fact that at 60 weeks, the Ca and P containing birefringent and electron-dense particles were observed only within giant cells, suggests that most of the β-TCP of the root replica were biodegraded and/or bioabsorbed by the host. Taken together, the evidence suggests that the Ca and P observed in the inclusion bodies of the giant cells were remnants of β-TCP that were eliminated by the host cells. These intracellular remnants of the β-TCP may not be of clinical significance, because the particles were within host cells that should be biologically eliminated in due course, or would be removed during surgical re-entry for placement of an osseointegrated implant.
CONCLUSIONS

The results of this study allow us to conclude that root replicas made out of $\beta$-TCP are biocompatible and bioabsorbable. Osseous healing occurred in both the test and control sockets, but the healing process appears to be delayed by the presence of $\beta$-TCP particles.

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