Bone tissue response to an oily calcium hydroxide suspension in tibial defects. An experimental study in minipigs.

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Conflict of Interests

The authors report no conflicts of interest related to this study.
Abstract

Bone tissue response to an oily calcium hydroxide suspension in tibial defects. An experimental study in minipigs.

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Objectives: The present study was conducted to evaluate bone formation after the application of an oily calcium hydroxide suspension (OCHS) into defects created into the tibial bone of minipigs.

Materials and methods: Standardized defects (2x1.5cm) were created into the bone marrow space of four 4 Goettinger minipigs’ tibiae. Defects in the test group (2 minipigs) were filled with OCHS (Osteora, DFS-Diamon, Riedenburg, Germany) whereas defects in the control group (2 minipigs) were filled with animal’s own venous blood. Animals were sacrificed after healing periods of 4 and 8 weeks. Tibias were dissected, soft tissues removed and processed for histological analysis. Digital images (x200) were evaluated using the software CellD (Soft Imaging System, Munster, Germany). The following histomorphometrical landmarks were identified: defect size, mineralized and non-mineralized tissue as well as residual OCHS.

Results: Healing was uneventful in all four animals. In the test group, new bone formation was observed in the vicinity of the defect margins whereas the centre of the defect was dominated by non-mineralized tissue. Mean percentages of mineralized tissue after 4 weeks were 23,01% in the test group vs. 43,45% in the control group. The mean value for residual OCHS was 7.11% at four weeks. After 8 weeks mean percentages of mineralized tissue were 28,15% in the test group vs. 44,39% in the control group as well as 7,05% for residual OCHS.

Conclusion: The application of both OCHS and venous blood contributed to early bone formation in this type of defect model. However, owing to the small sample size, these tendencies ought to be explored in further studies.
Introduction

In field of oral surgery, the regeneration of extensive bone defects resulting from trauma, tumors or osteitis still remains a challenge. Especially the reconstruction of the alveolar ridge allowing a rehabilitation involving endosseous implants requires demanding surgical approaches. At the present day, transplantation of either autografts, allografts, bone substitutes or distraction osteogenesis are routinely used regenerative techniques, each of them having important limitations concerning availability as well as biological or biomechanical drawbacks. Thus, the osteoinductive stimulation of bone formation has gained increasing interest. Especially the application of morphogenic and mitogenic growth factors and their effect on bone formation became subject to various studies [1,2]. Although several studies provided evidence that bone morphogenic proteins (BMP’s), growth/differentiation factor (GDF)-5 or platelet-derived growth factor (PDGF) may provide local bone augmentation [3,2,4], unpredictable results, treatment costs and uncertain safety arguments prevent a routine implementation of these approaches into daily practice. Thus, there is still a demand for alternatives for achieving a satisfying result of bone regeneration as well as the patients claim to a minimal-invasive and comfortable surgical therapy.

A promising input had been raised several years ago with the idea of using oily calcium hydroxide suspensions for tissue regenerative purposes [5]. Several experimental studies have shown that CH may possess antimicrobial [6] [7] and anti-inflammatory properties [8]. When applied on amputated dental pulp or into the root canal close to the apex, CH has been reported to result in a destruction of the vital tissue, leading to the formation of a necrotic layer and, subsequently, formation of a hard tissue barrier below the exposure site [9,10]. Additionally, CH also seems to have a positive influence on the healing of periapical lesions [11,12]. These effects may be mainly due to the alcalic properties of CH leading to a neutralization of the acidic metabolites of macrophages and osteoclasts [13]. However, the CH mechanism used to promote the repair of bone tissues may not only do so by providing rich Ca2+ and alkaline environment mineral deposition, but also by stimulating the calcification enzyme activity of osteoblasts [14]. So far, several groups have reported positive effects
in the treatment of periodontal defects [15] [16,17], but only little and controversial information is available about CH on bone regeneration [18] [19]. Up to now, effects of CH on osseous healing have been evaluated in open models [20] or external applications with GBR- techniques, not signalizing positive impacts [18] [19]. Yet, *in vitro* data indicate that oily CH-suspensions may have stimulous effects on cell growth, differentiation and metabolism of progenitor cells (Dietz & Bartholmes 1998 a, b). Kasaj 2007 et al. further observed that the addition of oily CH-suspension enhances mitogenic responses of human PDL cells by activating extracellular signal-related kinase (ERK1/2) and increasing cell proliferation [21].

To review the potential of CH on bone formation, the aim of the present study was to investigate the bone tissue response to an oily CH-suspension focusing especially on the process of resorption and bone formation using an animal model.

**Materials and Methods:**

**Animals**

Four Goettinger minipigs (weight between 15,5 kg and 19,5 kg) were used in the present study. The study protocol was approved by the Committee on Animal Ethics of the Victor Babes University of Medicine and Pharmacy Timisoara, Romania

**Surgical Procedure:**

All animals received premedication using 1% Atropin (0,04 mg/kg) by means of an intramuscular injection. Following intramuscular sedation with 2% Acepromazine (1,1 mg/kg) and 10% Ketamine (20mg/kg), anesthesia was initiated using Thiopental-sodium (4mg/kg) intravenously. During surgery, inhalation anesthesia was performed by the use of oxygen and Halothane (1-1,5%). To maintain hydration, all animals received 300-500 ml physiological saline solution at the end of surgery while anesthetized. Postoperative analgesia was ensured by intramuscular injection of Ketonal (0,05mg/kg). Control radiographs of the experimental sites were taken immediately after surgery.
None of the animals showed any disorder of movement postoperatively. Until the suture removal after 7 days, all animals were kept under observation in individual boxes before being held in one group. The temperature of the operated animals was maintained at an optimal value by setting the environment temperature at 22 degrees Celsius.

Following desinfection of the surgical area with Betadine, an incision penetrating skin, muscular layers and periosteum was performed in order to reflect a flap exposing the medial face of the tibia. Then, an osteosynthesis plate with three screw holes was placed over the medial tibial face to position the orifice of the central screw. Under continuous irrigation with sterile 0.9% physiologic saline a hole was drilled and the central screw was inserted marking the center of the future defect. By means of a diamond disk a rectangular cortical lid (2 cm length/1.5 cm width) on the medial plate of the diaphysis was created and carefully removed using a fine bone chisel. By this access, the bone marrow was entirely excavated including the arteria nutritia until a chamber of 2 cm/2 was established (Fig. 1a). To validate the exact planned volume, the excavation was continued until the defect could hold exactly 2 ml of sterile saline. The defects in the test group were filled with Osteora until the material started to spill (Fig. 1c). The defects in the control group were filled with the animal's own blood collected from the Arteria retroauricularis (Fig. 1b). Subsequently, the cortical lids were repositioned using the central titanium screw as well as two additional screws cranial and caudal of the defect allowing a stable fixation of the osteosynthesis plate (Fig. 1d). Wound closure was accomplished layer by layer using resorbable sutures (D-tek 3.0) and cutaneous single non-resorbable sutures (D-tek 3.0). Finally, all experimental sites were desinfected with a liquid disinfectant.

Animal preparation and retrieval of specimens

The animals were sacrificed after a healing period of 4 and 8 weeks, respectively, 2 animals (test group/control) were randomly assigned to each healing period. The euthanasia of the animals was performed by intracardiac injection of 20-40 ml of 20% Kaliumchlorid solution. The treated tibias were dissected from the limbs, the soft tissues were removed and the blocks containing the
experimental specimens were obtained. After removing the osteosynthesis plates, all specimens were fixed in 4% neutral-buffered formalin solution for 4 weeks.

**Histological preparation**

All specimens were dehydrated using ascending grades of alcohol and xylene, infiltrated and embedded in methyl-methacrylate (MMA; Technovit 9100 NEU, Heraeus Kulzer, Wehrheim, Germany) for non-decalcified sectioning. During this procedure, any negative influence of polymerisation heat was avoided due to controlled polymerization in a cold atmosphere (−4°C). After 20 h, the specimens were completely polymerized. The diaphysis of each tibia was sectioned transversally using a diamond wire saw (Exakt®, Apparatebau, Norderstedt, Germany). The sectioning was performed in the direction perpendicular to the long axis of the bone starting immediately at the end of the epiphysis and descending towards the diaphysis, up to the middle of the diaphysis below the bone plate covering the operated site. As a result, sections of approximately 500 μm in thickness were created. Subsequently, all specimens were glued with acrylic cement (Technovit 7210 VLC, Heraeus Kulzer) to silanized glass slides (Super Frost, Menzel GmbH, Braunschweig, Germany) and ground to a final thickness of approximately 40 μm. The specimens were stained with Toluidin Blue (TB; quality and quantity of mineralized tissue/newly formed bone).

**Histological analysis**

Histomorphometrical analysis as well as microscopic observations were performed by one experienced investigator masked to the specific experimental conditions. For image acquisition, a color CCD camera (Color View III, Olympus, Hamburg, Germany) was mounted on a binocular light microscope (Olympus BX50, Olympus). Digital images (original magnification ×200) were evaluated using a software program (CellID®, Soft Imaging System, Münster, Germany).

For the analysis, 6 sections of each experimental specimen, i.e. 2x Osteora and 2x control, were selected to be able to represent the entire dimension of the defect. As a result, a total of 24 sections were examined. The following histomorphometrical landmarks were analyzed within the sections:
defect size (DS), mineralized- (MT), non-mineralized (NMT) tissue and residual OCHS (OCHS) within the defect. Moreover, the reintegration of the cortical bone lid as well as new bone formation in the vicinity of the defect were examined.

Results

Clinical Healing

The postoperative healing was uneventful in all 4 animals. No allergies or foreign body reactions were observed throughout the whole study period. All wounds healed eventless and no signs of inflammation or infection occurred. After one week, all experimental sites revealed complete wound closure without any signs of local irritation.

Histological Evaluation

The mean values, standard deviation (SD) and medians for MT, NMT and OCHS in both groups after 4 and 8 weeks are presented in Table 1 and 2.

4 weeks

After 4 weeks, sections of both groups revealed a well reintegrated cortical bone lid indicated by newly built trabecular bone adjacent to the former gap of separation (Fig. 5a). In various sections of both groups an excessive bone formation around the cortical bone lid on the averted side of the defect close to the former attached titanium osteosynthesis plate was observed (Fig.5b). However, both groups contained sections showing non-mineralized defect gaps (Fig.5c). In test as well as control groups, tissue response was characterized by new bone formation close to the defect margins showing trabecular bone in the vicinity of the former defect margins, supporting fine branches of newly formed bone being orientated towards the centre of the defect (Fig. 2a,b).
However, in both groups the defect centre was dominated by non-mineralized tissue. In sections of the test group only small amounts of residual OCHS were detected being surrounded by a layer of mineralized tissue (Fig. 4a). Mean percentages of mineralized tissue were 25,51% in the test group vs. 42,44% in the control group. The mean value for residual OCHS was 7.11% at 4 weeks. After 4 weeks of healing no significances could be detected.

8 weeks

At 8 weeks, the maturation of bone adjacent to the defect margins into woven bone could be recognized in both groups as well as proceeding bone formation towards the centre of the defect (Fig. 3a,b). Concerning the bone formation above the cortical bone lid no differences could be observed at 8 weeks. In both groups, sections revealed a continuing bone formation originating from the defect margins showing solid branches of new bone towards the centre of the defect. Numerous specimens of both groups featured a dense network of trabecular bone supported by a solid layer of woven bone nearby the previous defect borders. However, in both groups only a couple of sections exhibited a dense bone formation inside the centre of the defect. Comparing both groups, the amount of mineralized tissue seemed to be higher in the control group, especially in the centre of the defect. In both groups the value for mineralized tissue only slightly increased between the healing periods 4 and 8 weeks. In the test group, the quantity of residual OCHS only slightly decreased indicating only minimal resorption between 4 and 8 weeks of healing (Fig. 4b). Mean percentages of mineralized tissue were 26,23% in the test group vs. 41,84% in the control group as well as 7,04% for residual OCHS. No significances could be observed after 8 weeks of healing.

Discussion

The present study was conducted to evaluate the tissue response to the application of an oily calcium hydroxide suspension (OCHS) in defects created into the bone marrow space of minipigs’ tibiae. The results of the present study illustrated that the application of OCHS did not promote bone regeneration after a healing period of four and eight weeks in comparison with the control group.
Histomorphometric and histological analysis indicated that in the test group quality and quantity of new bone formation tended to be inferior to the control group. These observations might be due to the chosen defect model. The configuration of this closed defect model served as an ideal setting for spontaneous healing with a cancellous environment surrounded by tibial bone marrow containing multipotent progenitor cells [22] [23] favouring bone regeneration in the control group. In the test group, the application of OCHS might have had an obstructive effect on early wound healing as it might have initially competed with blood clot formation within the defect. This assumption accusing OCHS of inhibiting angiogenesis in early stages of wound healing, especially when the resorption process of the grafting material has not advanced yet, is in accordance with the results of two other experimental studies evaluating OCHS effects applied in “closed models” in rats and dogs. The data illustrated obstructive effects of OCHS on bone formation in combination with GBR on cortical bones [18,19]. Stavropoulos et al. reported on OCHS interfering bone regeneration by showing only minimal new bone formation as well as nearly no signs of resorption [18]. Stavropoulos suggested that a reason for minor OCHS degradation was due to the compromised blood supply of the environment inside the capsules applied [18]. However, in the present study the histological analysis exhibited an obvious degradation process of the grafting material at 4 and 8 weeks after surgical application. The results presented no inhibition of bone formation, as observed in the previous studies with closed models, but showed lower amounts of mineralized tissue after 4 and 8 weeks in comparison to control sites. Hence, the obstructive effect of OCHS might have only affected the first phase of wound healing in this specific defect model resulting in delayed bone formation. These findings might indicate that the material could not fulfil the purpose of facilitating bone quantity in this specific defect model. However Ito et al. conducted a study in rats, comparing the healing in OCHS-filled extraction sockets with that in sockets left untreated to serve as controls. They reported that after 1 month of healing, sockets previously filled with OCHS showed statistically significantly larger amounts of bone fill than control groups (Ito et al. 2001). In another descriptive study [15], three-wall intrabony (4x4x 4mm) periodontal defects were produced bilaterally in a dog model and were randomly treated with either access flap surgery and the application of OCHS, or access flap
surgery alone. After 2 months of healing, larger amounts of regenerated bone and newly formed cementum were observed in the sites treated with OCHS than that found in the control sites, in which healing was predominantly characterized by the formation of a long junctional epithelium along the previously denuded root surface and only minimal bone regeneration [15]. The regenerative effects of OCHS in the mentioned studies representing open models could also be related to antibacterial effects of CaOH [24] - a result of the stable pH gradient of 7.5–9 - before being washed out of defects and pockets. Moreover, the results from in vitro studies showed beneficial effects of continuous OCHS presence on cell growth and differentiation of osseous and undifferentiated progenitor cells (Dietz & Bartholmes 1998 a, b; [25]. In order to gain additional data on the impact of OCHS on tissue response the present study was designed as an inductive trial in an ideal cancellous environment surrounded by tibial bone marrow containing multipotent progenitor cells [26,23]. However, the evaluation of the histological sections from the present experimental investigation revealed only a slight gain of osseous cell maturation and metabolism related to the application of OCHS. Thus, there seems to be a discrepancy between results observed in cell cultures compared to the application of OCHS in animal models. We can only speculate upon the reasons - one might be the absence of inflammatory cells in the in vitro studies [20]. However, CaOH is clinically successfully used in endodontics and has been identified to promote calcified tissue formation in various indications (e.g., dentin barrier, apical seal, apexification, radicular growth, healing of periapical lesions) [10]. Moreover, periodontal regeneration after OCHS application into pockets periodontal defects has been shown in experimental and clinical studies [25,16,15].

In the present study mean percentages of mineralized tissue at 4 and 8 weeks showed an inferiority of the test group regarding the amount of bone regeneration but without indicating statistical significant differences. This might me due to the low number of animals conducted in this study. Summarizing the results of the present investigation, the application of OCHS into closed bony defects showed good biocompatibility, decent resorption and new bone formation. Within the limits of the present study it can be concluded that OCHS might have a beneficial effect on new bone formation. The application of both OCHS and venous blood contributed to early bone
formation in this type of defect model. However, owing to the small sample size, an osteoinductive potential of OCHS ought to be explored in further studies.

Figure legends

Figure 1a. Empty defect configuration in the diaphysis of the tibia
Figure 1b. Control group filled with venous blood
Figure 1c. Defect filled with Osteora
Figure 1d. Repositioned cortical bone plate
Figure 2. Region of Interest (ROI)
Figure 3a. Test group after a healing period of 4 weeks
Figure 3b. Control group after 4 weeks
Figure 4a. Test group after 8 weeks
Figure 4b. Control group after 8 weeks

Figure 5a. Residual OCHS surrounded by mineralized matrix

Figure 5b. Residual OCHS surrounded by mineralized matrix (higher magnification view x 200)

Figure 6a. Both groups revealed a well reintegrated cortical bone lid indicated by newly built trabecular bone adjacent to the former gap of separation (Osteora group)

Figure 6b. Excessive bone formation around the cortical bone lid on the averted side of the defect close to the former attached titanium osteosynthesis plate (Osteora group)

Figure 6c. Section showing non-mineralized defect gaps (Control group)

Figure 7a. Mean MT values (in %) of test and control groups after 4 and 8 weeks

Figure 7b. Mean NMT values (in %) of test and control groups after 4 and 8 weeks

Figure 7c. Mean OCHS values (in %) of test group after 4 and 8 weeks

Figures
Fig. 7a
Tables

Table 1.
Mean values, SD and medians (in %) of NMT, MT and OCHS in test sites

<table>
<thead>
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<th>Group</th>
<th>Mean test</th>
<th>SD test</th>
<th>Median test</th>
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<td>NMT</td>
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<td>10,91</td>
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<td></td>
<td>MT</td>
<td>25,51</td>
<td>15,32</td>
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<td></td>
<td>OCHS</td>
<td>7,11</td>
<td>5,5</td>
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<tr>
<td>8 weeks</td>
<td>NMT</td>
<td>67,05</td>
<td>16,19</td>
</tr>
<tr>
<td></td>
<td>MT</td>
<td>26,23</td>
<td>12,45</td>
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<tr>
<td></td>
<td>OCHS</td>
<td>7,04</td>
<td>6,66</td>
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</table>

Table 2.
Mean values, SD and medians (in %) of NMT and MT in control sites

<table>
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<th>SD control</th>
<th>Median control</th>
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<tr>
<td>4 weeks</td>
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<td></td>
<td>MT</td>
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<tr>
<td>8 weeks</td>
<td>NMT</td>
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<td>23,19</td>
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<tr>
<td></td>
<td>MT</td>
<td>41,84</td>
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Literature


