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Osteopromotion with a plasmatransglutaminase on a β-TCP ceramic

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Abstract We investigated the osteopromotive properties of plasmatransglutaminase (F XIII), bone marrow and venous blood on a resorbable β-tricalcium phosphate (β-TCP) scaffold. A baseline binding and release study of F XIII from the scaffold showed a continuous release of 18% of the total dose after 48 h. The main study consisted of 18 adult sheep with cylindrical defects in both tibiae. The defects were filled with a β-TCP cylinder impregnated either with bone marrow, venous blood, F XIII or sheep were treated with 1250 IU F XIII intravenously over 14 days ($n = 4$ in each group). The defects were left open in two sheep. QCT and histology was performed after 6 and 12 weeks. The best bone ingrowth was seen after 6 weeks in the bone marrow group and after 12 weeks in the local F XIII group. The highest ingrowth on the inside of the cylinder proving the osteopromoting potential of F XIII was found in the local F XIII group. In our opinion F XIII is a good and readily available osteopromoting agent which can be used with β-TCP in cases of bone deficit to promote bone regeneration.

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Introduction

Bone regeneration and bone healing need an optimal environment consisting of a proper blood supply and a stable external or internal stabilization. These factors are promoting osteoblast ingrowth and proliferation. Whilst the golden standard in orthopedic surgery remains the autologous bone graft with optimal osteoconductive and osteogenic properties, current research is trying to mimic these effects by using different resorbable or non-resorbable scaffolds together with osteoinductive factors.

In the present study we investigated the behavior of a β-tricalcium-phosphate (β-TCP) implant and various osteopromoting substances. As non-autologous osteopromoting factor we used a transglutaminase (plasmatransglutaminase or F XIII). F XIII has several effects on cells. Primarily it causes a polymerization of fibrin monomers after activation by thrombin and leads to a stable blood clot [1]. Secondly, it stabilizes the cell membrane [2], stimulates cell proliferation, cell metabolism and cell adhesion [1, 3, 4]. In addition, the positive effect of F XIII on wound healing has been described in severe burns [5], abdominal surgery [6, 7], otorhinolaryngologic surgery [8] and pressure sores [9]. Furthermore, its beneficial effect on bone healing has been reported in animal and human studies for fracture healing, pseudarthrosis and implant fixation [10–14]. As autologous osteopromoting substances we used blood and bone marrow. Bone marrow and blood (especially blood-derived PRP) have previously shown to promote bone formation in clinical studies and both are known to be a source of osteopromoting and osteogenic agents [15, 16].

Materials and methods

In vitro study

As a baseline study we investigated the F XIII (Fibrogammin®) binding and release kinetics from the β-TCP cylinders (chronOS™, Synthes®). Nine β-TCP discs (8×10 mm) were impregnated with 10, 40 and 100 IU of F XIII (in triplicate) using a vacuum syringe. The discs were impregnated after the Synthes method [17] to allow an even distribution of F XIII. The discs were immersed in a Ringer bath and, under constant movement, the amount of F XIII released into the Ringer solution was measured (30 min, 1,2, 4, 8, 24 and 48 h) by ELISA. We used a special F XIII ELISA kit provided by ZLB Behring Research (Marburg, Germany).

In vivo study

We performed a bilateral tibial metaphyseal cylindrical defect of $\varnothing 8.5 \times 20$ mm in 18 adult sheep. An independent veterinarian prepared a large group of merino sheep (German Merino Landschafe) which were readied for several different studies. Preoperatively the veterinarian chose a random sheep out of this large group for our study in order to exclude any bias. However, only female adult sheep were chosen (average 4 years, average weight 61.5 kg). These critical size defects were filled with a β-TCP cylinder (chronOS™, Synthes®) in 16 sheep and left unfilled in 2 sheep (control group). The β-TCP cylinders were impregnated with autologous venous blood (group 1, $n = 8$) and autologous bone marrow aspirate from the sternum (group 2, $n = 8$). In group 3 ($n = 8$) the cylinder was inserted un-impregnated and a daily dose of 1250 IU F XIII (Fibrogammin®) administered iv. over 14 days. In group 4 ($n = 8$) the β-TCP cylinder was impregnated with 125 IU F XIII (Fibrogammin®). The blood and bone marrow aspirate impregnation was done under vacuum according to the chronOS™ manufacturers handling recommendations using a perfusion syringe [18]. The impregnation of the β-TCP cylinders with F XIII was also done under vacuum using a standard 10 mL Luer slip-tip syringe; vacuum was done by closing the tip with one thumb and oscillating the piston a few times. All sheep were immediately mobilized after surgery. No external or internal fixation was used. We observed no drop-outs.

Anesthesia protocol

Premedication with im. Detomidine was given after at least 12-h fasting. After sedation an iv. venous entrance was established in an auricular vene and Ketamine given intravenously. Subsequently, after orotracheal intubation the anaesthesia was maintained with inhalation anesthesia (isoflurane/O2 mix). All animals were observed post-op up to complete awaking in a with wood shavings interspersed single box. Post-op pain therapy was performed with iv. Metamizole for the first and sc. Metamizole for the following 4 days. Perioperative antibiotics were given to prevent infection.

Surgical protocol

After the preparation—shearing, skin disinfection and sterile draping—a 2 to 3 cm long incision was performed at the medial side of the prox. tibia. The bone defect was performed with the Synthes bone marrow harvesting set [19] and filled according to the investigational groups. With an average size of the tibial head in a full growth sheep of 46×38 mm (width $\times$ depth) the cylindrical defect was limited to 8.5×20.0 mm. The ChronOS™ cylinder was implanted at least 5 mm underneath the cartilages—bone border, in order to exclude an injury of the joint. Subsequently, the wound was closed with subcutaneous and resorbable skin stitches.

Animal care protocol

All animals were kept preoperatively over 1–2 weeks in playpens with straw for acclimatizing. Feeding took place with unlimited water and hay as well as rationed mixed provender. Claw care was performed if necessary.

Half of the sheep in each group were sacrificed after 6 and 12 weeks accordingly and histology of the explanted specimen was performed.

Euthanasia protocol

At the study time intervals the sheep were anesthetized with iv. Detomidine and Ketamine prior to euthanasia with T61®.

The metaphyseal tibial regions were explanted and fixed in 70% ethanol for 3 weeks. They were then dehydrated through an ascending alcohol gradient, replaced with xylene, and then embedded in methylmethacrylate. Once polymerized, the blocks were trimmed to fit into a Leica 1600 circular saw (Leica Microsystems AG, Glattbrugg, Switzerland) and cut to produce 200 μm thick sections. The mid-defect section was glued onto an opaque plexiglass slide, and then ground down and polished with an Exakt Micro-Grinding System (Exakt Apparatebau GmbH, Norderstedt, Germany) to obtain a final thickness of around 100 μm. The sections were then stained with Giemsa-Eosin and digitally recorded at a resolution of $1{,}300 \times 1{,}030$ pixels (Axiocam/Axiovision V3.1, Carl Zeiss, Oberkochen, Germany). Bone ingrowth and remaining chronOS™ cylinders were assessed by image processing (Zeiss KS400,

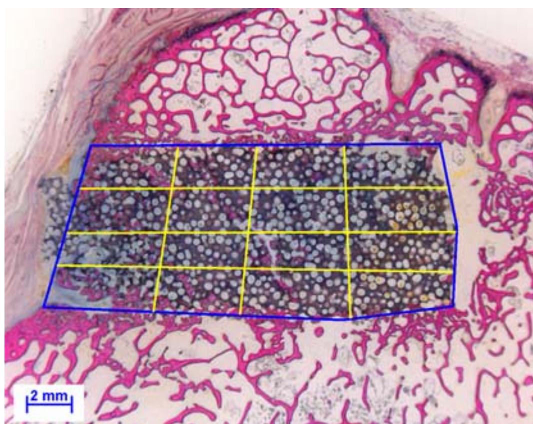

Fig. 1 Distribution of the specimen cut area into 4 inner and 12 outer squares for histological analysis

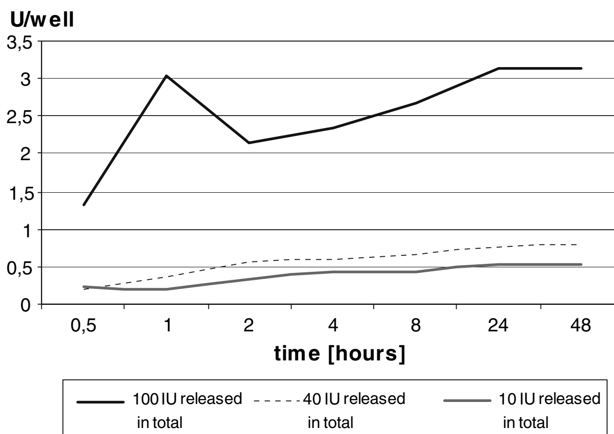

Fig. 2 F XIII release from β-TCP impregnated with 10, 40 and 100 IU, (n = 27)

Carl Zeiss, Oberkochen, Germany). Each slide was separated into 4 × 4 rectangular squares resulting in 12 outer and 4 inner squares (Fig. 1). The outer squares and inner squares were measured separately in order to differentiate bone ingrowth from the surrounding bone and cell mediated ingrowth from the inside of the cylinder. The analysis was performed by calculating the percentage of chronOS™, bone and non-mineralized tissue in relation to the total, inner or outer area accordingly.

The statistical analysis was performed by an independent statistical institute (Data Investigation Company Europe), using several ANOVA models. The analysis was performed separately for the week 6 and week 12 data. The difference between the tibiae was evaluated using repeated measures ANOVA with as factors treatment (five groups), tibia, and tibia by treatment interaction. The treatment effect was further evaluated on the basis of the means of the left and right tibiae, which were analyzed using ANOVA, with treatment as only factor. In case of an overall treatment effect (at the 5% level of significance), two by two comparisons were performed of the five groups.

The study protocol including operation, anesthesia and euthanasia of the animals was approved prior to the study by the local animal rights committee (Thuringia national office for food security and consumer protection, study number 14-02/02).

Results

In vitro study

We investigated the binding and release kinetics of F XIII on a β-TCP cylinder with 70% porosity. The ELISA results are shown in Fig. 2. We found that there is mainly a linear release of F XIII from the chronOS™ cylinder over time. The average rate of release per hour depended on the primary concentration and ranged from 0.05 IU/h (primary total concentration 10 IU) and 0.08 IU/h (primary total concentration 40 IU) to 0.34 IU/h (primary total concentration 100 IU) within 48 h. At that time point an average of 18% of the total dose was released.

In vivo study

Histological analysis

The results of the histological analysis are shown in Figs. 3–5. The values of remaining chronOS™, non-mineralized tissue (NMT), newly formed bone for the total area and the inner and outer squares are shown separately. We found the best bone formation after 6 weeks in the bone marrow group 2 (Fig. 3). This result is also shown in the analysis of the outer and inner squares (Figs. 4 and 5). In all other treatment groups we found no difference in

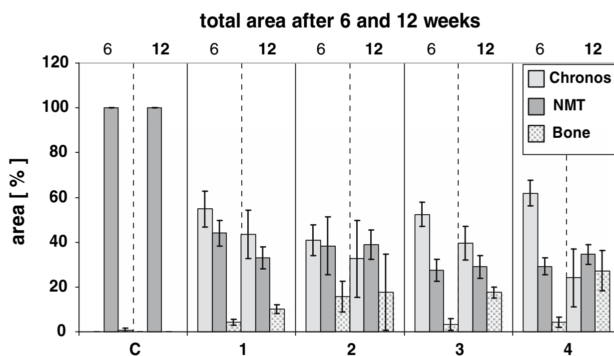

Fig. 3 Measurement of ceramic (ChronOS), non-mineralized tissue (NMT) and bone ingrowth (Bone) at 6 and 12 weeks in the control group and in the experimental groups in % of the total area after 6 and 12 weeks

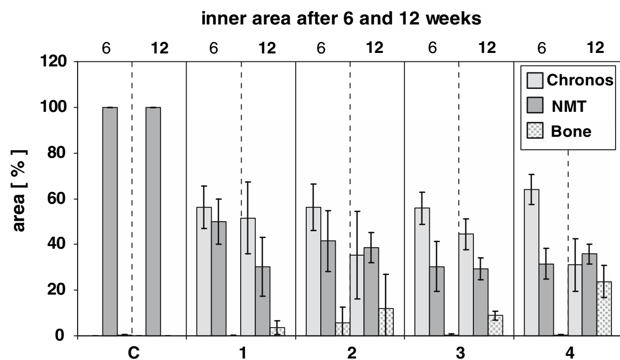

Fig. 4 Measurement of ceramic (ChronOS), non-mineralized tissue (NMT) and bone ingrowth (Bone) at 6 and 12 weeks in the control group and in the experimental groups in % of the inner area after 6 and 12 weeks

bone formation. However, the F XIII groups 3 and 4 have considerably improved in bone formation after 12 weeks. In fact, group 3 shows at 12 weeks a comparable bone formation to group 2 (17.7 versus 17.6%) and a much better bone formation than group 1 (9.7%). Interestingly, after 12 weeks we found the best bone formation in the local F XIII application group 4 (27.2%, Fig. 3). Comparing bone formation at the inner and outer surface of the scaffold, again we observed that group 4 performed better than the others (Figs. 4 and 5).

The analysis of the β-TCP resorption, bone formation and the amount of NMT shows that at 6 and 12 weeks the bone formation is in balance with the resorption of the β-TCP and changes accordingly in all groups. The amount of NMT, however, tends to stay the same over the time in all groups (Figs. 3–5).

The control group shows only 0.8% bone formation at 6 weeks and no measurable bone formation after 12 weeks.

In conclusion, the bone marrow group and the local F XIII group show the best bone formation after 12 weeks.

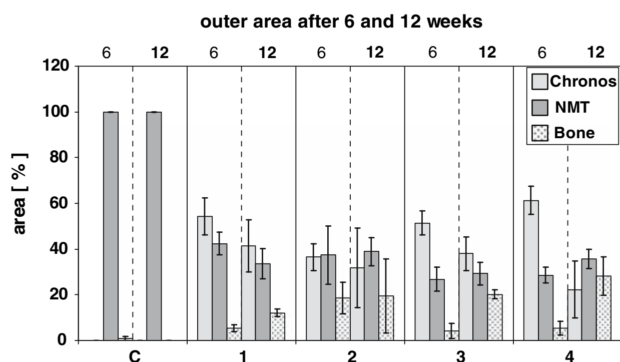

Fig. 5 Measurement of ceramic (ChronOS), non-mineralized tissue (NMT) and bone ingrowth (Bone) at 6 and 12 weeks in the control group and in the experimental groups in % of the outer area after 6 and 12 weeks

The bone formation is highest in the local F XIII group, especially on the inside of the β-TCP cylinder. The least bone formation after 6 and 12 weeks is found in the venous blood group 2.

The histological slides demonstrate a good resorption of the β-TCP cylinder in group 2, 3 and 4 (Fig. 6). The structure of the newly formed cancellous bone mimics that of β-TCP and is denser than the surrounding cancellous bone.

The bone defect of 8.5 mm on the tibial plateau was considered as a critical size bone defect as no sheep showed a spontaneous bone healing during the follow-up period (see Figs. 3–5 and 7).

Statistical results

Significant statistical differences were found between the bone ingrowth of all investigational groups versus control (p at 6 weeks < 0.001, at 12 weeks < 0.003). At week 6 the bone marrow group shows significant bone ingrowth in comparison to the venous blood group ($p = 0.01$) and the F XIII groups (local $p = 0.006$, iv $p = 0.005$). At week 12 no statistical difference between the bone marrow and the F XIII groups was observed ($p = 0.455$).

The separate analysis of the outer and inner surfaces shows a highly statistical bone ingrowth at the inner surface of the local F XIII group ($p = 0.004$) compared to all other groups. This result is also reflected in Fig. 4. All other groups did not show any significant bone ingrowth on the inner surface of the cylinder between week 6 and 12.

Discussion

This study was designed to investigate the osteopromotive properties of F XIII in a critical size bone defect. The potential osteopromoting properties of F XIII were tested against current alternative clinical approaches such as venous blood or bone marrow impregnation on a synthetic and resorbable β-TCP scaffold. Our results, considering the low number of animals, indicate that F XIII groups show similar bone formation at 12 weeks compared to the bone marrow impregnation group.

We have chosen a critical size defect on the tibial head to demonstrate the changes in the scaffold.

The concept of a critical size defect has been described often in the literature [20–24] but the dimensions vary greatly depending not only on the species but also on the different bones and bone structures. It has been shown that the defects on animals with intact periosteum lie between 5 mm and 4 cm. As those defects per definition do not heal spontaneously, the remodeling, osteoinductive or

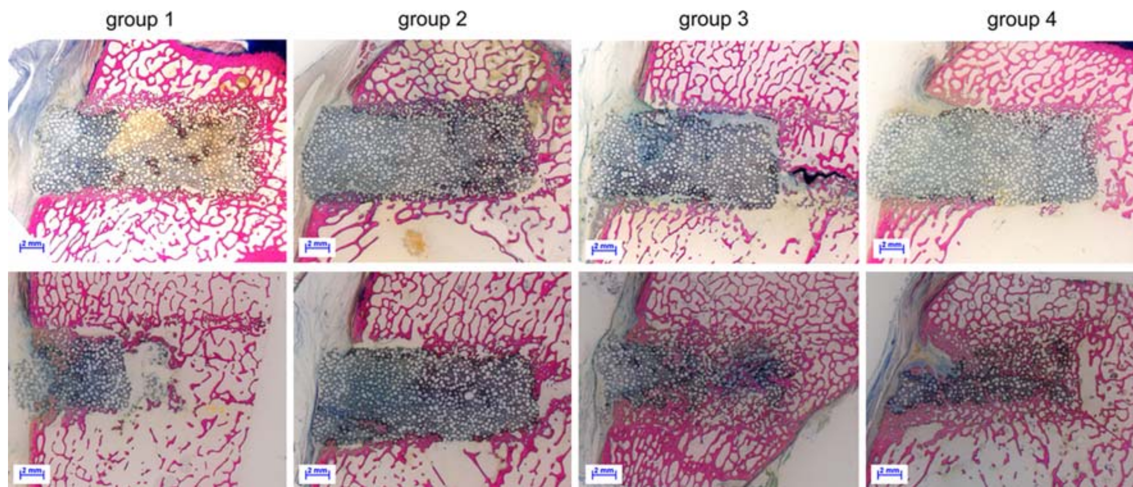

Fig. 6 Histology of bone ingrowth in the investigation groups; upper row results at 6 weeks; lower row results at 12 weeks

osteoconductive interaction of implants can be assessed. The implant as such is necessary in those cases to allow vascular and cellular ingrowth.

Several factors contribute to the remodeling of the implanted scaffold. The operation as such creates a bony defect, which activates new bone formation and the release of growth factors. The defect alone, however was large enough (e.g. critical size) not to cause bone healing in those cases where we didn't implant a scaffold (Fig. 7). Furthermore the defect consisted on one site of cortical bone and on all other sides on cancellous bone. Those cancellous parts with their bone marrow and abundance of angiogenic and osteogenic cells are the condition sine qua non for proper bone healing [23, 25, 26]. We could show that the β-TCP implant serves as optimal osteoconductive material, as it allows not only optimal ingrowth from cells whilst implanted in a cancellous bone section, also we could show in our wrongly implanted scaffold (Fig. 8) that the scaffold showed local bone ingrowth at the contact area to the cortical bone. It is furthermore well known that non-vital bone grafts are resorbed by osteoclasts and replaced by lamellar bone [27–29]. These changes were not observed in our groups demonstrating that the chosen animal model was well suited for this study.

In the initial kinetic baseline study we found a linear release of F XIII from the β-TCP scaffold. About 80% of the total dose remained within the scaffold after 48 h. By extrapolating this finding we can assume that the F XIII release continues up to a maximum of 10 days, which will be similar to the main F XIII treatment period in patients (e.g. 5–14 days) [7]. The daily dose administered to patients with bone healing related problems is generally 1250 IU iv. per day [9, 12, 13]. Therefore, the dose of 100 IU present within the scaffold probably even exceeds the needed local amount of F XIII for an optimal stimulation. The exact action of F XIII is still not known, and it is difficult to explain the observed delay in bone formation at week 6 in both F XIII groups compared to the bone marrow group. Interestingly, at 12 weeks the bone formation levels in the local F XIII group, and to a less extend in

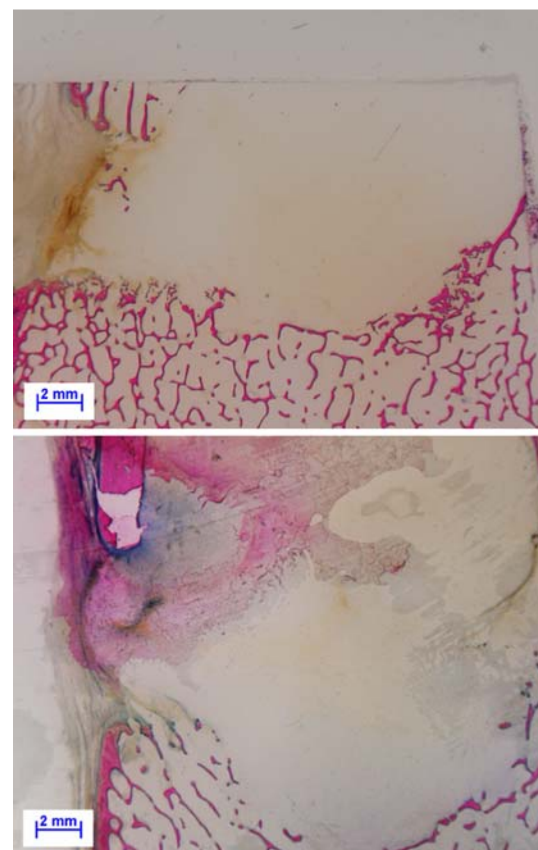

Fig. 7 Histology of bone ingrowth in the control group C; upper row results at 6 weeks; lower row results at 12 weeks

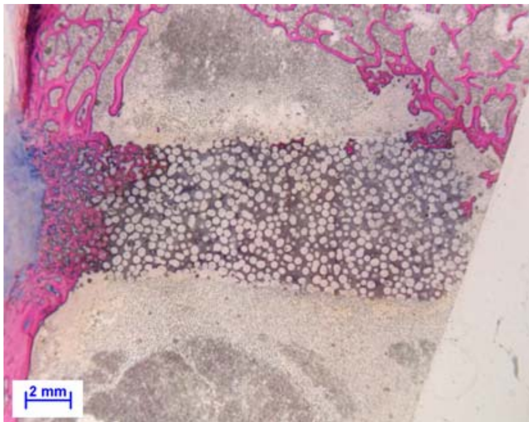

Fig. 8 Misplacement of cylinder in the medullar cavity of group 2 at 12 weeks. Note the good bone formation at cortical level

the F XIII iv. group, showed similar values as in the bone marrow group. This was reflected by a steep increase in bone formation in both F XIII groups from week 6 to 12.

Furthermore, the analysis of the outer and inner squares, which was originally performed to show an ingrowth of bone from the surrounding bone tissue toward the inside of the cylinder, showed in the inner squares twice as much bone in the local F XIII group as in all other groups. The evaluation of the outer squares also showed that the best bone formation was found in the local F XIII group, although, the difference was less pronounced. This higher bone formation in the inside of the implant may be induced by F XIII as it has been shown that an organized fibrin matrix resulted in a higher activity of the local stem cell population, which is essential for the cell proliferation and differentiation phases in bone healing [3, 13].

The comparative low bone formation in the bone marrow group, with only 2% more bone formation at 12 weeks than after 6 weeks, is a consequence of a misplacement of the scaffold during surgery into the intramedullary cavity, which can be clearly seen in Fig. 8, fortunately misplacement occurred only in one sheep. Without these outliers the overall results for this treatment group would have shown a much better outcome. This can be seen in the high standard deviation in Fig. 3. As in week 6 all four samples show a better bone formation than the outliers in week 12, it is reasonable to assume that we could have expected a higher bone formation at 12 weeks in the bone marrow group than the present one.

F XIII has been shown to have several other interesting properties. First, it can stabilize the blood clot, which is the basis for a proper wound healing [3]. Second, at the cellular level, the mitogenic effect on osteoblasts and osteoblasts precursor cells as well as the stimulation of alkaline phosphatase activity have been reported [4, 13, 30]. Furthermore, in in-vivo studies, F XIII has shown a higher tensile strength of the healed bone segment in sheep [10, 11]. In another study, it was observed a statistically significant positive effect of F XIII on implant fixation and ingrowth compared to the control group [14]. Finally, drill holes on the tibial diaphysis were performed in sheep and filled with autologous bone graft [31]. Following iv. F XIII treatment, the authors found a significant higher remodeling of the transplant site in 92% of the treated animals compared to 79% of the control group. Thus the beneficial action of F XIII as osteopromoting agent in bone healing has been observed in several large animal studies, while only one study performed on rats showed no difference between treatment and control group [32]. In our study, due to the low number of animals, our statistical analysis had an overall small power which only picks up very important changes or differences. Those were found in the bone marrow group and the F XIII groups concerning the bone ingrowth at the inner surface of the cylinder. Those significant results were found notwithstanding the small sample size. The ANOVA test we used is especially designed for those data [33].

Our study also indicated that the β-TCP scaffold was replaced by bone with time. Locally there is a denser cancellous bone than in the surrounding bone (see Fig. 6), which in theory may support higher stresses. We assume that this newly formed bone will later be subject to the remodeling process according to Wolff's Law as a result of its complete integration into the adjacent bone under physiological loads and shear stresses.

Apart from autografts, allografts and xenografts, synthetic bone substitution materials are becoming increasingly popular. These materials are favored because of the absence of disease transmission due to their synthetic origin and the needed of a second surgical intervention. The use of ceramics, especially calcium phosphates (CaP), is motivated by the fact that the inorganic component of bone is hydroxyapatite (HA, $Ca_5(PO_4)_3OH$). However, being the most stable Ca–P in term of solubility, HA can be regarded as non-resorbable within years [34]. On the other hand, the Ca–P ratio of β-TCP ($Ca_3(PO_4)_2$) makes it suitable for osteoclast resorption, it is slower resorbable than α-TCP, but much faster than HA [35]. ChronOS is resorbed on the cellular level by osteoclast and macrophages without developing an inflammatory reaction [36]. In addition, the chronOS™ scaffold used in this study, with its open-porous and interconnecting structure, allows blood vessels as well as the host tissue to gradually grow into the pores [37]. The three-dimensional structure with a 70% interconnecting porosity and a pore size between 100 and 500 µm allows a good ingrowth of blood vessels and non-mineralized tissue [18, 36].

Conclusion

The impregnation of a porous osteoconductive bone substitute with autologous bone marrow resulted in an increased bone formation supporting the osteopromotive properties of this approach. We could in addition show that F XIII is also enhancing bone formation, both locally and iv. with possibly a better effect of the local administration. This study has also several limitations, such as the small group size and the lack of any biomechanical analysis. Therefore, this study should be considered as a preliminary investigation, which will require further experimental research to confirm the potential role of F XIII as an osteopromoting agent.

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