Reconstruction of bone defects using a novel bone graft substitute

PhD Thesis

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1. Introduction

Despite modern bone grafting techniques, management of large segmental bone defects remains a significant surgical challenge. Bone defects develop several different ways: bone fracture can end up in the occurrence of a pseudoarthrosis in 5-10 % of the cases, high energy trauma, prosthesis revision arthroplasty, treatment of musculoskeletal infections or excision of bone tumors can also result large bone deficiencies.

The significant development seen in musculoskeletal surgery brought reconstructive approach into focus. Amputation was tolerated previously by patients if acceptable functional result had been provided, in recent years however, anatomical reconstruction of the affected limb became substantial besides the functional restoration. This progress made bone grafting very popular: some 2.2 million grafting procedures are performed each year worldwide. This outstanding number made bone – after blood – the second most commonly transplanted tissue.

The sore need to find new bone graft materials has driven musculoskeletal research to develop novel bone substitutes. An ideal bone graft should feature good mechanical strength and significant osteoinductive, osteoconductive and osteogenic capabilities, should be available in large quantities at a reasonable price. Traditionally, human bone derived grafts were used, many different osteoinductive materials appeared later on in an attempt at improving bone healing. Bone morphogenic proteins (BMPs), demineralized bone matrix (DBM) and platelet rich plasma (PRP) are the most widely studied ones. Bone morphogenic proteins are known to have a notable osteoinductive effect, but they are rather expensive and a suitable carrier material for their optimal application is still missing. Though demineralized bone matrix provided encouraging results in experimental setups, the concentration of BMPs in DBM products is highly variable leading to unreliable clinical outcomes. Being a natural source of growth factors, the use of PRP is a reasonable option. However, recent studies questioned its efficacy and reported its limited ability to enhance bone healing. In addition, the exact mode of action of PRP is unknown and a few studies suggested that a significant proportion of its effect is attributable to other components than platelets or growth factors.

Some other materials have also been tested, and are in use in the everyday practice, such as cell-based substitutes, ceramics and polymers. (1. table)

Table 1.

Different bone substitutes by Laurencin.

Csontpótló szerek csoportosítása
A. Harvested bone grafts and graft substitutes
I. Bone grafts
1. Autogenous bone grafts
2. Bone marrow
3. Allogenic bone grafts
II. Demineralized bone matrix (DBM)
B. Growth factor-based graft substitutes
I. BMPs and other growth factors
II. Platelet-rich plasma
C. Cell-based bone graft substitutes
I. Stem cell
II. Collagen
III.: Gene therapy
D. Ceramic-based bone graft substitutes
I. Calcium hydroxyapatit
II. Tricalcium phosphate
III. Bioactive glass
IV. Calcium sulphate
V. Injectable ceramic cements
E. Polymer-based bone graft substitutes
I. Natural or synthetic polymers
II. Degradable or non-degradable polymers
F. Miscellaneous
I. Coralline HA

In spite of the significant efforts to find an appropriate bone substitute none of the above listed has gained a general acceptance in everyday use. Therefore our aim

was to find a bone graft that is immunologically inert, has reasonable capacity to promote bone healing, inexpensive and easily available in large quantities.

2. Objectives

The gold standard critical sized model has been criticized in recent times, among others because it is designed to test bone defects, where the regenerative process fails to bridge an oversized gap. However, the orthopedic surgeon commonly faces smaller defects with compromised healing capacity. In these situations the critical size model has limited applicability, instead, an experimental setup is needed, which is clinically more relevant and allows the investigation of smaller bone deficiencies without a critical size gap. Our aim was to develop a novel bone defect model where compromised bone healing can be tested.

Previously our workgroup proved that albumin coating of bone allografts allowed mesenchymal stem cell adherence and proliferation. Stem cells did not cover the surface in a monolayer but rather span the pores of the graft. Our secondary objective was to test the novel graft in vivo in our newly developed bone defect model.

3. Material and methods

3.1. Interposition femoral defect model

Adult male Wistar rats (n=26) of 459 to 692 grams were housed and maintained at 12/12 day/night cycles and were provided with water and lab chow ad libitum. All animal procedures were approved by the Scientific Research Committee of the Semmelweis University. The animals were separated into four groups, the experimental protocol is summarized in Fig. 1. In group I. the classical critical size defect model was established. A 6 mm thick osteoperiosteal defect was created in the femur of 8 rats. The bone was fixed by plate and screws. The animals were sacrificed after 4 weeks. Group II. was chosen to observe the normal regenerative capacity of

the bone in case of a 2 mm defect. Therefore, after plate and screw fixation a 2 mm mid-diaphyseal osteoperiosteal femoral segment was removed in six animals, and the defect was left empty. The animals were euthanized after four weeks. In group III. a 2 mm mid-diaphyseal osteoperiosteal defect was created in the femur of 6 animals. After fixing the bone, a 2 mm thick bone cement spacer was interposed into the defect to block normal bone healing. The animals were sacrificed after four weeks. In group IV. the interposed spacer was taken out after 4 weeks and the defect was left empty for 4 more weeks; these 6 animals were sacrificed after 8 weeks. The issue in this group was to investigate whether the bony defect will regenerate after 4 weeks if it is left alone.

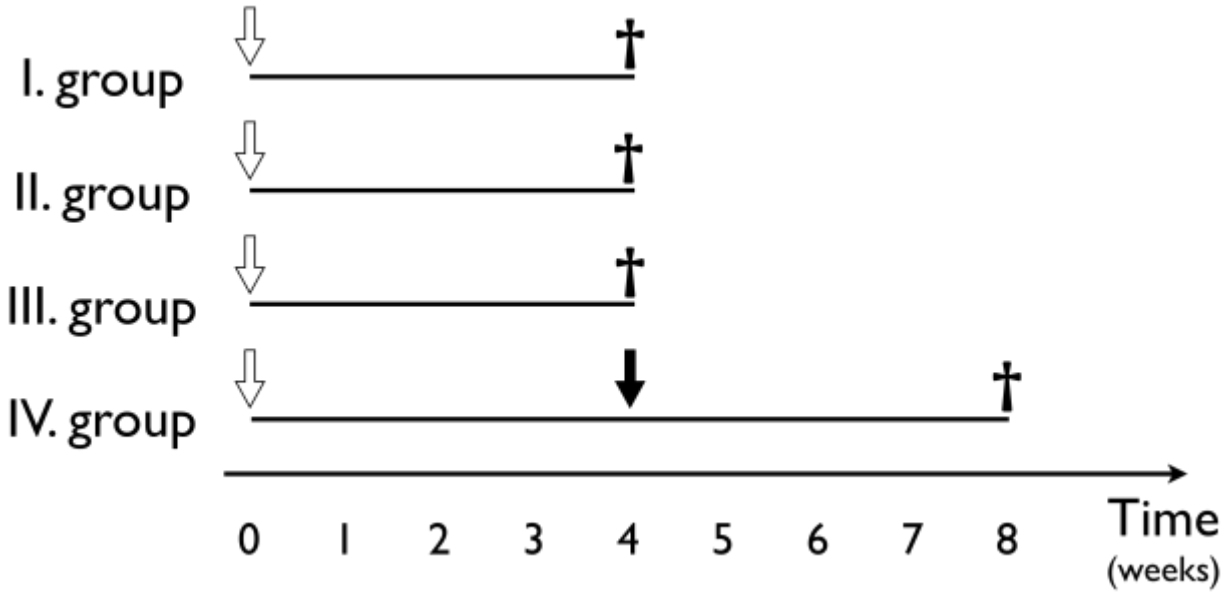

Figure 1.

The four groups of animals. White arrow (⇩) indicates the time of the first procedure in each group. Black arrow (🡻) shows the time of the removal of the spacer in group IV. Black cross (†) represents the time when the animals were sacrificed.

In vivo imaging and image analysis

Radiophosphonate imaging using ^{99m}Tc-methylydene diphosphonate (^{99m}Tc-MDP, Skeleton®, Medi-Radiopharma Ltd, Hungary) and SPECT/CT imaging with a NanoSPECT/CT imaging system (NanoSPECT/CT®, Mediso Ltd-Bioscan Inc, Hungary-US) was performed weekly until the 4th week. One of the most widely used radiopharmaceuticals, ^{99m}Tc-MDP is considered to accumulate in sites of elevated osteoblast activity and thus can be used both in clinical settings and in experimental animals to evaluate and quantitatively characterize osseous regenerative processes.
The animals were scanned using the four-headed quantitative multiplexed multipinhole NanoSPECT/CT system, 3 hours after the injection of approximately 80 MBq ^{99m}Tc-MDP. After image acquisition, data were reconstructed with the instrument's dedicated HiSPECT software for multiplexing multi-pinhole projections. Images were subsequently analyzed using the InVivoScope (Bioscan Inc., US) and the Fusion (Mediso Ltd., Hungary) image analysis software packages. Volumes of interests (VOIs) were defined in the reconstructed 3-dimensional volumes. The isotope activity distribution within the VOI was then summed to determine the uptake of related tissues. Radioactive dose concentration of ^{99m}Tc-MDP was determined by dividing measured radioactivity in all animals (in MBq) with the whole body weight (in g) of the animal. This radioactive dose concentration was used to divide the lesion VOIs' radioactive concentration in each animal. A Standardized lesion radioactive uptake value (SUV) like parameter was calculated according to Blake.

Ex vivo μCT analysis

After harvesting the femora, new bone formation and the development of a union or non-union was evaluated using a μCT scanner (Skyscan 1172 X-Ray microtomograph, Kontich, Belgium). The system has a 5 μm spot size, scans were carried out with 40 kV applied voltage without filter. All cross sections contained 1024x1240 pixels with an isotropic voxel size of 10 μm. Analysis of the specimens was done with the CT Analyzer 1.10.1.0 software (SkyScan, Kontich, Belgium), 3D visualization was performed using SkyScan CTvox software (SkyScan, Kontich, Belgium). Bone specimens were placed in a cylindrical sample holder so that the

longitudinal axis of the bone was parallel to the sample holder. A high resolution scanning was made with slice number up to 1700 depending on the length of the specimen. Results proximal and distal to the osteotomy site and in the osteotomy region was evaluated according to Verna and Schmidhammer.

A cylindrical volume of interest (VOI) was placed between the screw holes neighboring the osteotomy site. The VOI was positioned in such a way that the base of the cylinder started at the distal end of the proximal screw hole and ended at the proximal end of the distal screw hole on the other side of the defect. This way the column incorporated the diaphyseal section of the femur between the screw holes together with the osteotomy site. To be able to investigate new bone formation in the defect, inside this total VOI three regions were determined. After visualizing the margins of the original defect, the 1st sub-VOI was set from the proximal screw hole to the osteotomy site, the 2nd sub-VOI covered the original defect itself, and the 3rd sub-VOI was located from the defect until the distal screw hole. Since the removed bone segment differed in the different groups, the length of the 2nd sub-VOI was determined to be 2 mm in group II., III. and IV., and 6 mm in group I. Thus, sub-VOI 1 and 3 represented the original bone stock of the femur and sub-VOI 2 referred to newly formed bone in the osteotomy site. The set of bone area/tissue area (B.Ar/T.Ar) values were determined slice by slice for each sub-VOI. Bone regeneration in the defect was related to the original bone stock, hence it was characterized by comparing the difference in the B.Ar/T.Ar values of sub VOI 2 and that of sub VOI 1 and 3. This difference is referred throughout the present study as “relative bone volume”. Union / non-union was assessed using 3D reconstruction according to Schmidhammer. In order to separate mineralized elements from the background, a fixed threshold was set. Union was defined as a continuous bony bridge across the defect site.

Histology

After radiologic examination the cut out femoral bones were formalin-fixed, decalcified, embedded in paraffin and sectioned longitudinally. The slides were stained with hematoxyline and eosin and examined with light microscopy.

3.2. The in vivo role of serum albumin in bone regeneration

All animal procedures were approved by the Scientific Research Committee of Semmelweis University. Adult male Wistar rats (n=39) of 496 to 692 grams were housed and maintained at 12/12 day/night cycles and were provided with water and lab chow ad libitum. The animals were separated into six groups summarized in Table 2. In the first half of the animals the classical critical size model was established by creating a 6 mm wide midshaft defect which was either left empty or filled by an uncoated or albumin coated graft. In the second half of the animals non-union model was applied by blocking bone healing by a spacer. The interposed spacer was removed after 4 weeks and the osteotomy gap was either left empty or filled by uncoated or albumin coated bone graft. The detailed technique of the albumin coating of the grafts was described previously. Each animals were euthanized four weeks after the implantation procedure.

Table 2.

Experimental protocol.

	I. group	**II. group**	**III. group**	**IV. group**	**V. group**	**VI. group**
Model type	Critical size	Critical size	Critical size	Inter-position	Inter-position	Inter-position
Size of defect	6 mm	6 mm	6 mm	2 mm	2 mm	2 mm
Graft type	Empty (controll)	Uncoated	Albumin coated	Empty (controll)	Uncoated	Albumin coated
Number of animals	7	5	7	6	6	8

Surgical technique, ex vivo μCT analysis and histological examination are detailed above.

4. Results

4.1. Interposition femoral defect model

No complications were observed during or after surgery. Healing progressed uneventfully in all animals. The bone cement spacers and the plate and screws were not dislocated, superficial or deep wound infection was not observed.

In vivo NanoSPECT/CT analysis during the healing process showed that the epiphyseal parts of the femur at both the operated and intact sides had similar osteoblast activities, which can serve as a control for the defect site. Radiopharmaceutical uptake was slightly increased at the defect site compared to the intact parts of the diaphysis, however, it did not reach the epiphyseal levels. Differences were most prominent one week after surgery, and did not change significantly during the course of the study.

The classical critical size model in group I. had a relative bone volume of **-7.85±1.47 %** with a 12.5 % union rate (1 out of 8). In group II., which served as control osteotomy group, we measured **a -2.47±0.88 %** relative bone volume in the defect site with a 83.33 % union rate (5 out of 6). In group III, where a spacer was interposed into the defect non-union developed in all cases, and callous formation did not stabilize the spacer in place. The relative bone volume was **-7.9±1.06 %** in the defect site. However, in group IV., where the spacer was removed and the bone was left to heal for a further 4 weeks a bone defect occurred in 5 out of 6 cases (83.33 %) and the relative bone volume remained at a low level (**-4.73±1.36 %.**, Fig. 2.). Histological analysis confirmed bony consolidation of the defect in cases of a union, while an essentially bone-free zone was microscopically seen at the defect site in cases of non-union determined by μCT. The relative bone volume was significantly ($p<0.05$) lower in groups I and III compared to that of group II.

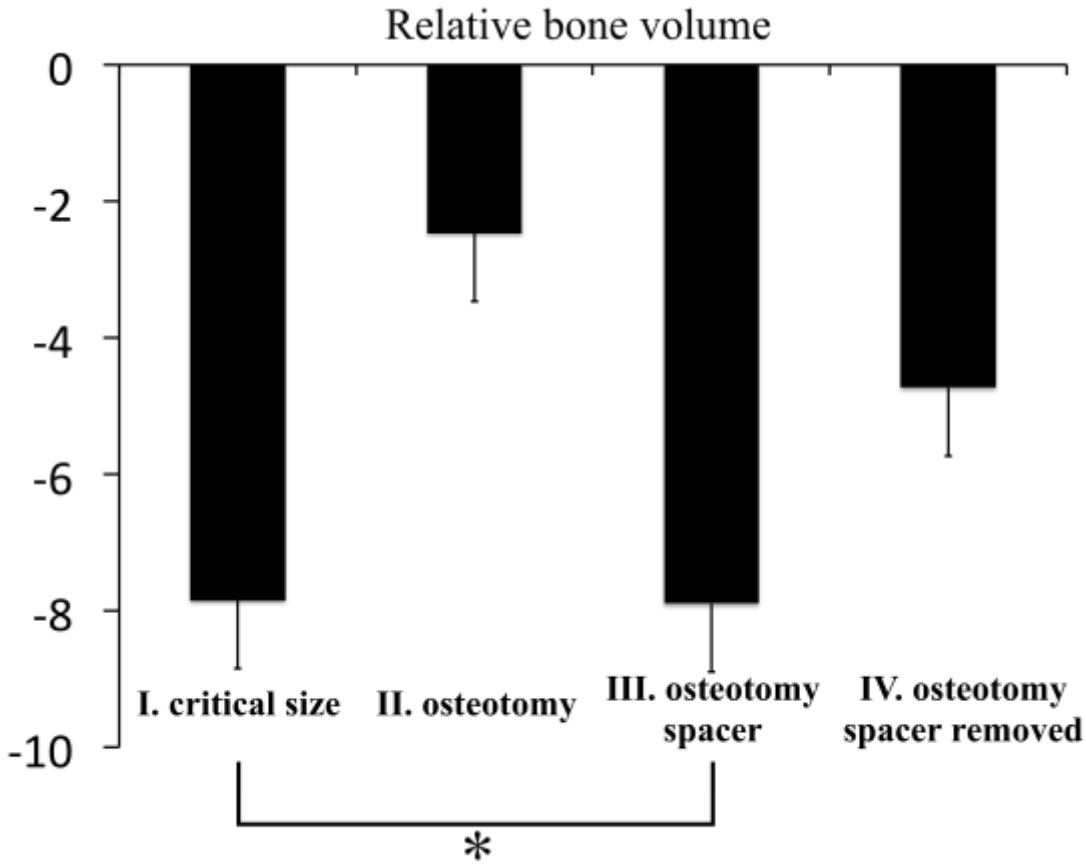

Figure 2.

Relative bone volume is presented on the chart. In group II. relative bone volume is significantly higher than in group I. and III. ($p<0.05$)

4.2. The in vivo role of serum albumin in bone regeneration

No complications were observed during or after surgery. Healing occurred uneventfully in all animals, graft dislocation or infection was not observed. The analysis of the 3D μCT reconstructions of the graft-host interface allowed the distinction of 3 categories of non-union (Fig. 2). The graft either resorbed completely (resorption), or remained visible without attachment to any of the bone ends indicating sequestration (sequestration) or attached to one side of the defect (adherence). Trabecular separation values clearly identified each case where the graft was resorbed (Fig. 3).

In the critical size model, where a relatively large gap was filled by a bone graft, healing was not observed in any of the groups either if the defect was left empty or whether it was filled by any graft type. The relative bone volume in the defect site was comparable among the 3 groups. In this model graft resorption was the most prominent outcome without consolidation of the defect.

When the non-union model was used and the osteotomy gap was left empty, all of the animals developed a non-union (union rate 0/7) and relative bone volume in the defect site was **-6.93 % ± 2.1 %.** If the osteotomy gap was filled with an uncoated bone graft, the union rate slightly increased to 2/6 and relative bone volume was **-8.36 % ± 0.87 %**. However, when an albumin coated bone graft was applied the union rate increased markedly to 6/8 with a relative bone volume of **-10.92 % ± 3.74 %** (Fig. 3). Micromorphometry data showed that trabecular thickness and trabecular pattern factor increased significantly when albumin coating was applied, further supporting the finding that albumin coating beneficially affected the bone remodeling in the defect.

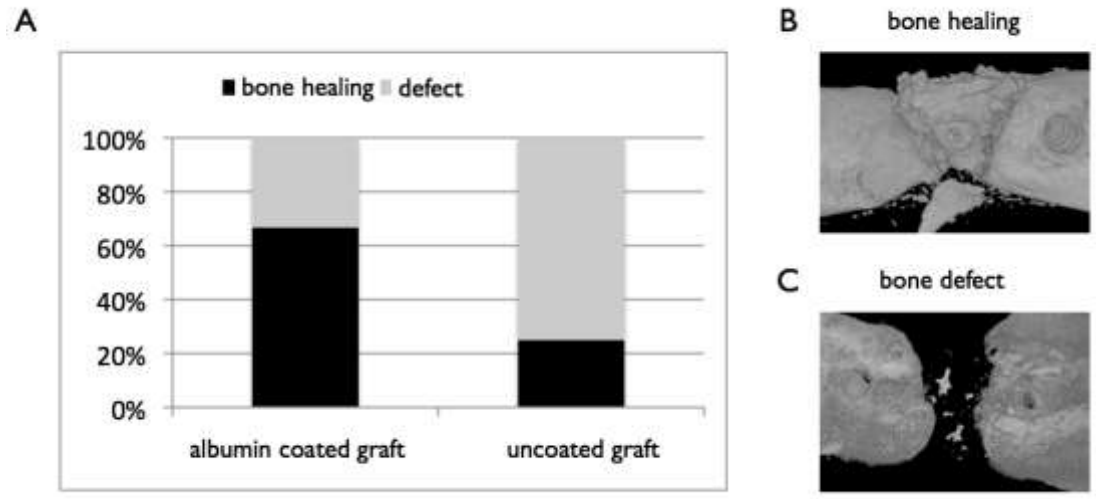

Figure 3.

Albumin coating of bone grafts resulted in 75 % of bone healing versus the 33 % healing rate of uncoated grafts. (A). 3D μCT reconstruction of bone healing (B) and bone defect (C) can be seen on the right side.

5. Conclusion

5.1. Interposition femoral defect model

Our results showed, that a dependable femoral segmental model can be achieved by using the interposition technique, furthermore, even when the spacer was removed bone buildup at the defect site was decreased indicating that this anatomical location and the applied surgical procedure provide a suitable model for compromised bone healing. The plate fixation technique provided reference points for both the implantation of bone graft candidates and the imaging of the regeneration process. Since the model was developed in small animals and the surgery is easily standardized, it allows economical screening of novel materials.

5.2. The in vivo role of serum albumin in bone regeneration

Our results showed that albumin coating of freeze-dried cancellous bone grafts improved significantly the remodeling characteristics of the graft in a non-union model. This improvement was so prominent at the macro level that it achieved consolidation of a non-union in most cases where the uncoated grafts failed. The clinical significance of this result is that an easily applicable factor, serum albumin, can enhance the efficacy of bone grafting from failure to load-bearing callous.

6. List of publications

6.1. Publications related to the thesis

1. Gábor Skaliczki, Miklós Weszl, Károly Schandl, Tibor Major, Miklós Kovács, József Skaliczki, Heinz Redl, Miklós Szendrői, Krisztián Szigeti, Domokos Máté, Csaba Dobó-Nagy, Zsombor Lacza: Compromised bone healing following spacer removal in a rat femoral defect model. Acta Physiol Hung 2012 Jun; 99(2): 223-232 , IF: 0.821

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