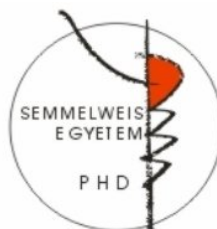


# **Investigation of the effect of freeze-dried human serum albumin on the biocompatibility of cancellous bone allograft**

Ph.D. Thesis

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## **1. Introduction**

The replacement of segmental bone defects is still a challenge for orthopaedic surgeons, especially when the self-healing ability of the bone is compromised. Under such circumstances bone grafts often fail to incorporate into the host tissue leading to the development of nonunion. Currently, autogeneic bone grafts are known as the best for the replacement of bony defects because they are immunologically identical to the host, thus their incorporation is more secure than that of materials of foreign origin. Furthermore, the autogeneic bone grafts have innate biological and biophysical cues that are supposed to facilitate their fast incorporation. In spite of the beneficial properties the extensive clinical use of autografts is not possible owing to their limited availability, therefore there is an increasing need for donor bone (allograft) as a potent alternative of the autografts. Chemical sterilization and antigen extraction followed by freeze-drying of bone allografts is a beneficial preparation method to reduce the risk of disease transmission and graft-versus-host reaction, thereby ensure the safe clinical use of the allografts. However, the chemical treatment may deteriorate the biological utility of the bone allografts reducing their biocompatibility with host cells and tissues that compromises their osseointegration, eventually.

## **2. Objectives**

The main objective of the present doctoral work was to investigate and improve the *in vitro* and *in vivo* biocompatibility of chemically sterilized, antigen extracted, freeze-dried human bone grafts.

Specific questions were:

- 2.1. Does the chemical treatment detrimentally affect the *in vivo* biocompatibility of freeze-dried human bone grafts?
- 2.2. What coating substance and method would be sufficient to enhance the biological value of chemically sterilized, antigen extracted freeze-dried human bone grafts?
- 2.3. How would the coating influence the *in vitro* and *in vivo* biocompatibility of the freeze-dried human bone grafts?

### 3. Materials and methods

#### 3.1. Investigation of the *in vivo* biocompatibility of chemically sterilized, antigen extracted freeze-dried human bone graft

The animal experiment had been approved by the Local Committee of Animal Research Ethics. Male Wistar rats (Toxi-Coop, Hungary) weighing 500–600g were anesthetized with 1.5 L/min oxygen, 200cm<sup>3</sup>/min halothane (Sigma Aldrich, St Louis, MO). The tail was washed three times with braunol (Braun Medical, Bethlehem PA) and ligatured at the tail root for the prevention of bleeding. The tip of the tail was surgically removed after which a standardized defect was created by drilling through the distal side of the caudal vertebrae (C4-C5) by using a custom made drill with 2 mm diameter, and with a shoulder at 3.5 mm to ensure a standardized depth. To prevent the self-regeneration of the vertebrae a stainless steel spacer was implanted into the drill hole. The wound was sutured and the animals were returned to their cages. After 12 weeks, the animals were anesthetized and the spacer was replaced. The cavital defect was filled either with PMMA (Heraeus Palacos R; n=5), premixed calcium phosphate cement (pCPC; n=5), Sr-doped calcium phosphate composite spheres (SrCPS; n=5), with impacted chemically sterilized, antigen extracted human freeze-dried bone chips (West Hungarian Regional Tissue Bank; n=5) or left empty (n=7), and

the wound was closed. At this time point a third group of animals (n=5) was added to serve as a positive control. Within this group, a defect was created as previously described, but the defect was left to heal normally without any spacer. The wound was closed by the same procedure as mentioned earlier. Twelve weeks later, all animals were over-anesthetized and euthanized by exsanguination. The last two vertebrae (last operated vertebra plus one healthy vertebra) were fixed in 4% formaldehyde and analyzed with  $\mu$ CT (Skyscan 1172 X-ray micro- tomography Skyscan, Kontich, Belgium) and histology.

The  $\mu$ CT scans were carried out applying a 60 kV voltage and an Al-filter. Reconstruction was done with a modified Feldkamp algorithm using Skyscan Nrecon software. The  $\mu$ CT reconstruction was obtained by rotating the view through 180 degrees (rotation step 0.5 degrees). SkyScan CTvox (Kontich, Belgium) was used for the 3D visualization.

Formaldehyde fixed vertebrae were decalcified by immersing them into Biodec-R solution for 1 week. Five micron longitudinal sections were cut from the paraffin blocks and mounted on glass slides. Conventional hematoxylin-eosin (Merck & Co) staining was used to confirm the results of the  $\mu$ CT measurements.

### **3.2. *In vitro* experimental design**

Three types of bone grafts, such as chemically sterilized, antigen extracted human cancellous bone allograft (West Hungarian Regional Tissue Bank), lyophilized bovine bone (BioOss, Geistlich Pharma AG) and synthetic hydroxyapatite (META BIOMED) were divided into three main experimental groups, i.e. control, Test A and Test B groups (Table 1).

Preparation of the chemically sterilized, antigen-extracted freeze-dried allografts: the cadaveric bones were washed in methanol for 4 hours,

then they were digested in a solution of 0.1 M phosphate buffer saline, 10mM sodium-azide and 10mM monoiodoacetic acid for 24 hours. Next, the bones were subjected to partial decalcification using 0.6 M HCl at room temperature for 4 to 6 hours. The as-produced human bone grafts were sterilized in ethylene-dioxide at 27°C, then they were freeze-dried aseptically (primer drying: 32°C, 2Pa, 12h; second drying: 32°C, 0Pa, 12h).

Test A and Test B groups were further divided into subgroups according to the method of coating, such as aqueous coating and freeze-dried coating, respectively. As coating substances albumin of human serum origin (200g/1000ml, BIOTEST), fibronectin of human serum origin (20µg/ml, Sigma Aldrich), and 1,5% porcine type I collagen (Biom' up) were used. The albumin was diluted in 1:2 using phosphate buffered saline. As control, uncoated bone grafts were used in the experiments. In Test group A, the bone grafts were soaked into the aqueous solution of either human serum derived albumin or fibronectin or collagen and incubated at +4°C overnight. Upon the elapse of the incubation period the bone grafts were removed from the protein solutions and placed into cell culture dishes where mesenchymal stem cells (MSCs) were seeded onto their surfaces instantly. In Test group B, bone grafts were incubated overnight in aqueous solutions of the same proteins, as it was detailed in the case of Test group A. However, after overnight incubation the bone grafts were removed from the aqueous protein solution and they were freeze-dried at 32 °C, at 1 Pa for 24 hours. After freeze-drying the bone grafts were placed into cell culture dishes where cells were seeded onto their surfaces immediately.

Samples before cell seeding were taken from each batch of Test A and Test B groups for mechanical and optical characterization. For mechanical characterization the Vickers hardness test method was applied,

while scanning electron microscopic images were acquired for structural characterization. The HV Vickers – hardness measurement is performed with a 136° angle of the vertex and square based diamond-pyramid. Flat surface areas were selected for micro-hardness measurement where the diamond-pyramid was pressed into with 50 g load weight for a period of 5 seconds. On each sample at least five measurements were carried out, while the two diagonals of the impression and the micro-hardness values were measured and averaged. The numeric value (HV) of the Vickers – hardness was determined based on the following formula: the load force (F) explicit in Newton (N) was divided by the surface area (A) of the impression in mm<sup>2</sup>, and then the result was multiplied with a constant (C = 0,102).

The surface characteristic of the bone grafts was investigated by scanning electron microscopy (SEM) (Philips XL 30). An argentiferous adhesive was applied on the bottom of the samples that were coated with an electrically conductive gold layer using a vacuum-pulverisation method. The procedure was then performed in vacuum. The photographs were taken in the secunder electron (SE) mode with 15kV accelerating voltage. The secunder electrons are able to emerge from the uppermost layers, having a thickness of 5-50 nm, meaning that they are extremely sensitive to the disproportionate surface. The full surface area of the samples was investigated and representative microscopic images were acquired at 50x, 200x and 1000x magnifications.

**Table 1. *In vitro* experimental groups.** In Test group A, the bone grafts were immersed into the aqueous solution of either human serum derived albumin (A) or fibronectin (B) or collagen (C). In Test group B, the bone grafts were incubated overnight in the aqueous solutions of the same proteins, which then were freeze-dried onto the surface of the bone grafts ( $\alpha$ ,  $\beta$ ,  $\gamma$ ).

	<b>Allograft</b>	<b>BioOss</b>	<b>Hydroxyapatite</b>
Control	Uncoated	Uncoated	Uncoated
Test A	<b>A)</b> Aqueous albumin	<b>A)</b> Aqueous albumin	<b>A)</b> Aqueous albumin
	<b>B)</b> Aqueous fibronectin	<b>B)</b> Aqueous fibronectin	<b>B)</b> Aqueous fibronectin
	<b>C)</b> Aqueous collagen	<b>C)</b> Aqueous collagen	<b>C)</b> Aqueous collagen
Test B	<b><math>\alpha</math>)</b> Freeze-dried albumin	<b><math>\alpha</math>)</b> Freeze-dried albumin	<b><math>\alpha</math>)</b> Freeze-dried albumin
	<b><math>\beta</math>)</b> Freeze-dried fibronectin	<b><math>\beta</math>)</b> Freeze-dried fibronectin	<b><math>\beta</math>)</b> Freeze-dried fibronectin
	<b><math>\gamma</math>)</b> Freeze-dried collagen	<b><math>\gamma</math>)</b> Freeze-dried collagen	<b><math>\gamma</math>)</b> Freeze-dried collagen

Isolation of mesenchymal stem cells: Human bone marrow samples were obtained from young patients (aged 2-20) during standard orthopaedic surgical procedures, with the informed consent of the patients or their parents under approved ethical guidelines set by the Ethical Committee of the Hungarian Medical Research Council. Only such tissues were used that otherwise would have been discarded. The bone marrow was taken into T75 flasks and diluted with Dulbecco's Modified Eagle's Medium culture medium containing 10% foetal calf serum (FCS), 100 U/ml penicillin and 10  $\mu$ g/ml streptomycin, 2mM L-glutamine and 1g/l glucose. The flasks were incubated at 37 °C in a fully humidified atmosphere of 5% CO<sub>2</sub> for 3 days. After the incubation period the bone marrow derived mesenchymal stem cells (BMSCs) adhered to the surface of the flasks, the remaining components of bone marrow were eliminated by washing with phosphate-buffered saline. The used BMSCs were between passages 1 and 5 in the experiments. The BMSCs were labelled with the fluorescent membrane dye Vybrant DiD (excitation/emission: 644/665 nm, Molecular Probes, Invitrogen, USA) for 30 minutes at 37°C in monolayer.

The protocol that we followed for the isolation and culturing of dental pulp derived mesenchymal stem cells (DPSCs) was based on the procedure of Gronthos *et al*, however it was implemented with minor modifications in our experiment. Human impacted third molars were collected from adults (18–26 years of age). The tooth was cut around the cemento-enamel junction by sterile dental fissure burs to expose the pulp chamber. The pulp tissue had been removed from the crown and the roots then digested in a solution of collagenase type I (3 mg/ml) and dispase (4 mg/ml) for 1 h at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 µm strainer and were seeded into 6-well plates with alpha modification of Eagle’s medium (α-MEM) supplemented with 20% FCS, 100 µM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, then grown under standard cell culture conditions. As opposed to BMSCs, the DPSCs did not take up the Vybrant DiD dye to allow uniform staining, thus their proliferation was followed up with UV-VIS spectrophotometer (BIOTEK Powerwave XS) using Alamar Blue assay (Biosource, Invitrogen, USA).

The lineage specificity of cells was confirmed by the presence of lineage-specific cell surface markers with flow cytometry (BD® FACS Calibur, Becton Dickinson, NJ, USA). Haematopoietic lineage-specific surface markers (CD34, CD45) and mesenchymal surface markers (CD73, CD90, CD105 and CD166) were investigated.

The mesenchymal stem cells (MSCs) were seeded onto the surface of the bone grafts by two methods, i.e. A) under standard cell culture conditions; and B) under dynamic cell culture conditions:

A) The MSCs had been trypsinized and suspended in culture medium then applied with pipette to the surface of the test and control bone grafts (100.000 cells per graft). After seeding, the cells were expanded on



the bone grafts under standard cell culture conditions for 18 days and their proliferation was investigated at the 3<sup>rd</sup> and 18<sup>th</sup> days.

B) First, 100.000 cells per scaffold were seeded on the surface of bone grafts and stored under standard culture conditions for 24 hours. Following the incubation period, the bone grafts were placed into a bioreactor tube, which had been filled with 25 ml cell culture medium comprising 1,5 million MSCs in suspension. The bone grafts had been incubated in bioreactor under dynamic cell culture conditions for 24 hours then the cells were further expanded on the surface of bone grafts under standard culture conditions for 18 days. The viability and quantity of attached MSCs on the surface was investigated after 3 and 18 days of incubation.

The survival of the fluorescent dye labelled BMSCs was observed with confocal microscopy (LSM 510 META, Zeiss) on the surface of control and test groups. Three individual view fields were randomly selected on the surface of the grafts where the quantity of pixels belonging to the fluorescent BMSCs was measured.

The cell culture medium of DPSCs was supplemented with 10w/w% AlamarBlue containing medium, which other components were identical to the normal culture medium of the cells. The bone grafts were incubated in this medium for 4 hours in cell culture incubator under standard cell culture condition. After incubation 200 µl of supernatant was pipetted into 96-well plate and the absorbance was measured at 570 nm and 600 nm wavelengths.

Test A and Test B subgroups (Table 1) were investigated in an observational study with longitudinal design where the mutual exposure was cell seeding onto the surface of bone grafts and the survival (effect of the exposure) of the seeded cells was measured at two time points during the

experiments, i.e. 3<sup>rd</sup> and 18<sup>th</sup> days. The possible outcomes of the exposure were classified according to pre-set verification criteria that also constituted the basis of the evaluation of the performance of the bone grafts in the course of the *in vitro* study (Table 2). Only those grafts were further investigated *in vivo* animal studies that facilitated the long-term survival and proliferation of the MSCs in the *in vitro* experiments.

Repeated measures one-way ANOVA analysis was performed (Tukey's post hoc test) to compare the quantity of MSCs on the scaffolds. A p value < 0.05 was considered significant.

**Table 2. Verification criteria to evaluate the *in vitro* performance of coated bone grafts.** Those bone grafts were progressively excluded from further investigations that did not facilitate the adherence or proliferation compared to their uncoated counterparts. Those bone grafts were excluded from further experiments that fell into at least one exclusion category.

	<b>Baseline</b>	<b>Decrease</b>	<b>Stagnate</b>	<b>Increase</b>
Adherence	Quantity of attached cells on uncoated bone grafts	Decrease in the quantity of adhered cells compared to baseline <i>Exclude</i>	Does not affect the quantity of adhered cells compared to baseline <i>Exclude</i>	Increase in the quantity of adhered cells compared to baseline <i>Include</i>
Proliferation	Proliferation rate of cells on uncoated bone grafts	Decrease in the proliferation rate compared to baseline <i>Exclude</i>	Does not affect the proliferation rate compared to baseline <i>Exclude</i>	Increase in the proliferation rate compared to baseline <i>Include</i>

### 3.3. *In vivo* biocompatibility study of coated human bone grafts

The Institutional Review Board of Semmelweis University approved the surgical protocol of the animal study. The investigation of the *in vivo* biocompatibility of the coated bone grafts was carried out in a nonunion model, where the self-healing potential of the bone was compromised. Adult male Wistar rats (n=39) weighing 496–692g were housed and maintained at 12/12 day/night cycles and were provided with

water and lab chow ad libitum. The animals were anaesthetized with halothane in a 1:1 mixture of N<sub>2</sub>O and O<sub>2</sub>. The surgical site on the thigh around the femur was shaved and disinfected. The skin, the subcutaneous layer and the fascia were incised, the tensor fascia latae, and the vastus lateralis muscles were separated from the biceps femoris muscle. The femur was exposed from the hip joint to the knee, with special care to preserve the periosteum. A 5 hole steel plate (Mini plate; Sanatmetal, Eger, Hungary) was fixed to the diaphysis of the femur by four 1.5 mm wide and 8 mm long cortical screws (Sanatmetal, Eger, Hungary) using two proximal and two distal holes, while leaving the middle hole empty. After fixing the plate by the screws, an osteoperiosteal segment was removed at the level of the middle hole using a reciprocating saw (Electric Pen Drive, Synthes GmbH, Oberdorf, Switzerland). The bone was cut precisely through both cortical layers together with the periosteum. The size of the defect was 2 mm, where a preformed thick surgical-grade sterile PMMA (Heraeus Medical, Wehrheim, Germany) bone cement spacer was interposed for four weeks. The PMMA spacer was secured to the plate by 3-0 non-absorbable sutures to avoid displacement. Then, the muscle lobes were stitched by 3-0 interrupted absorbable sutures over the femur, and the skin was closed by 3-0 interrupted nylon sutures. After 4 weeks a second procedure was performed to allow bone grafting. In the first step, the sutures were removed and the femur was exposed as previously described. Then the PMMA spacer was removed and the defect was either left empty, or filled with an uncoated or a freeze-dried albumin coated bone human bone graft block. After graft insertion the wound was closed in the same manner as detailed earlier. Following 4 weeks the animals were sacrificed by exsanguination under anaesthesia and the femora were harvested. The development of a

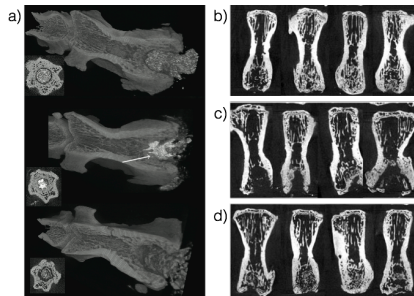
union/nonunion was evaluated by  $\mu$ CT (Skyscan 1172 X-Ray microtomograph, Kontich, Belgium).

## 4. Results

### 4.1. *In vivo* biocompatibility of chemically sterilized, antigen extracted freeze-dried human bone graft

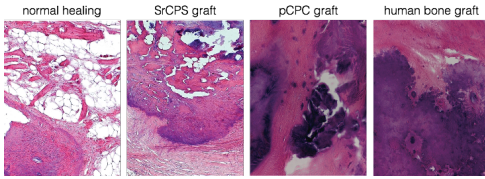
Figure 1 (upper image on Panel a) shows that the defect was completely filled with PMMA and no bone formation was observed. In the vertebrae filled with human bone chips no or slow resorption was observed, and limited bone healing (bottom image on Panel a). In case of pCPC, in the vertebrae (middle image on Panel a) new bone formation was observed (white arrow). Bone defects without any spacer or filling showed good bone regeneration with new trabecular bone being formed in each sample providing a defect union rate of 100% (Figure 1, Panel b). In the delayed healing group, where the spacers were removed at 12<sup>th</sup> weeks and the defect was left empty for an

additional 12 weeks, bone formation was observed in three defects while no or very limited bone formation was observed in the remaining four defects, where the union rate was 43% (Figure 1, Panel c). The  $\mu$ CT images demonstrated



**Figure 1:** shows the representative micro-CT images after 12 weeks of the operated vertebra.

that trabecular bone was formed in the defects filled with SrCPS with a union rate of 80% with four defects being completely filled with new bone while in one specimen bone was formed, but it did not fill the entire defect (Figure 1, Panel d).



**Figure 2: Representative hematoxylin and eosin stained histological section of the distal end of rat-tail vertebra after 12 weeks of normal healing and SrCPS, pCPC and human bone graft implantation.**

The histological assessment showed that there was some bone formation in the bone chip group; however the bone chips are still demarcated from the bone.

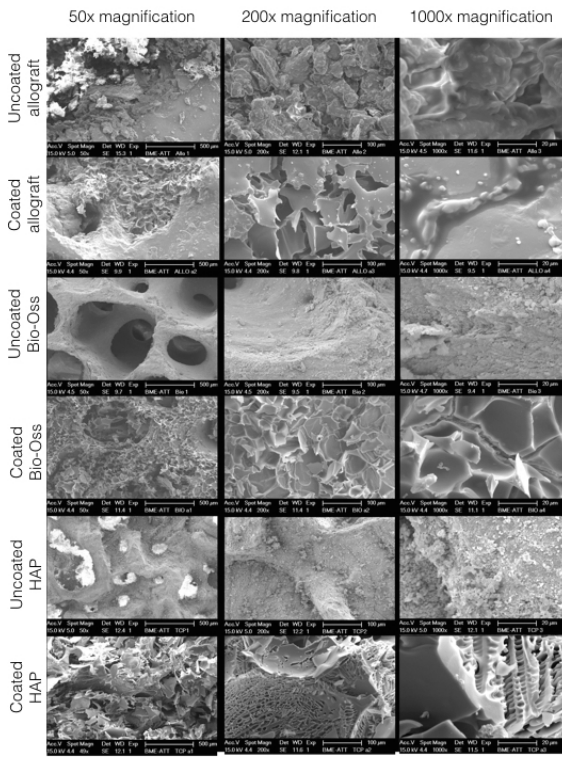
In the histology image bone chips are seen to have direct contact with newly formed bone (Figure 2). In contrast, the histological assessment confirmed that the SrCPS and pCPC had good biocompatibility and at the time of observation there were no visible signs of remaining SrCPS particles at light microscopy level indicating that the material was resorbed and built into the newly formed bone stock (Figure 2).

#### **4.2. Physical characteristics of coated bone grafts and *in vitro* adherence and proliferation of MSCs thereon**

The SEM analysis revealed significant differences in the macro-, and microstructure of freeze-dried human bone allograft, hydroxyapatite and lyophilized bovine bone (BioOss) (Figure 3). Hydroxyapatite exhibited the most compact structure with low number of micro-pores. In contrast, the texture of lyophilized bovine bone was rich in large diameter channels. The freeze-dried cancellous allogeneic bone graft exhibited segmented surface with pores of various sizes. The coating of the surface of bone grafts with freeze-dried albumin amorphous protein flakes masked the original structural differences. The albumin flakes showed the most homogeneous distribution on the surface of Bio-Oss, whereas the continuity of the albumin flakes was disrupted with random uncoated areas on allografts.

The xenogenic BioOss showed the lowest Vickers-hardness (14,9 HV  $\pm$  4,1). The allogeneic bone graft showed higher values (55,1 HV  $\pm$  7,7)

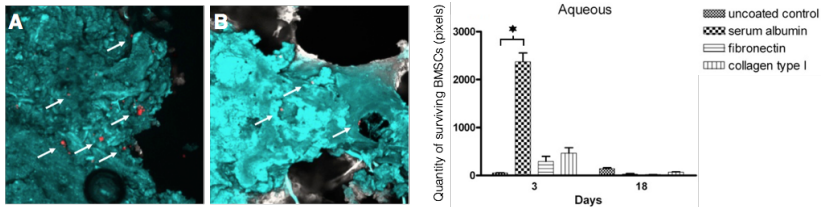
than BioOss, whereas the synthetic hydroxiapatite was a magnitude harder than the other two grafts ( $320,4 \text{ HV} \pm 44,6$ ). The micro-hardness values of freeze-dried albumin coated and uncoated allogeneic bone grafts were very similar ( $55.1\text{N} \pm 7.7$  vs.  $53.9\text{N} \pm 7.9$ , respectively).



**Figure 3. Macro- and microstructure of freeze-dried albumin coated and uncoated bone grafts.** Scanning electron microscopy shows significant differences in the texture of allograft, hydroxyapatite (HAP), and lyophilized bovine bone graft (BioOss). Hydroxyapatite has the most compact structure with low porosity. On the other hand, high connectivity and thin wall-thickness typifies BioOss. The structure of human bone allograft is different from the other two. It is more compact than BioOss and its surface contains multiple micro-pores. By coating the surface of bone grafts with albumin amorphous protein chips mask the apparent differences in the micro- and nanostructure.

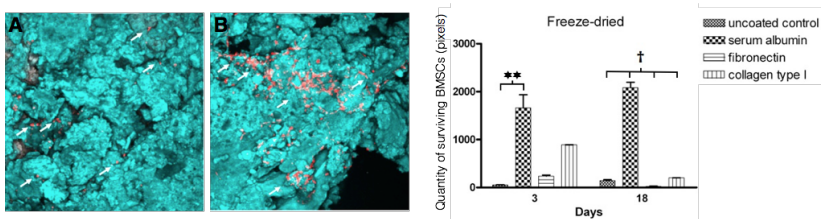
The coating of the surface of allografts with aqueous collagen or fibronectin slightly increased the initial adherence of BMSCs compared to the uncoated control, but the quantity of the cells was decreasing between the 3<sup>rd</sup> and 18<sup>th</sup> days of the experiments (Figure 4). In contrast, the albumin

coating of the surface of allografts markedly improved the initial adherence of BMSCs, however the cells disappeared from the surface by the 18<sup>th</sup> day (mean of pixels at the 3<sup>rd</sup> day:  $2373 \pm 142$ ; at the 18<sup>th</sup> day: 0)



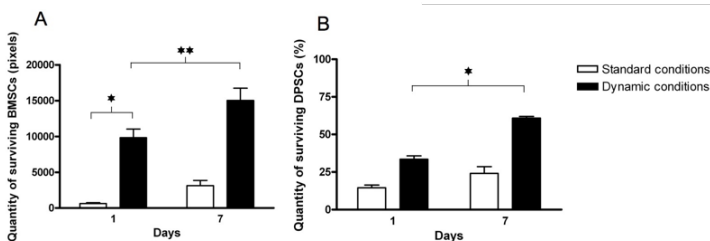
**Figure 4. Adherence and proliferation of BMSCs on the surface of allogeneic bone grafts coated with aqueous proteins.** The bar diagram shows that coating of allografts with aqueous albumin resulted in high cell density at day 3 ( $p^* < 0.05$ ), whereas fibronectin and collagen slightly increased the initial cell adherence. Irrespective to the composition of aqueous coating proteins only a few cells were detectable on the surface of allografts at day 18. Panels A-B show representative confocal microscopic images of uncoated allografts (blue) with Vybrant-DiD labelled BMSCs (red). The few observed cells on the surface at day 3 diminished even more by the 18<sup>th</sup> day.

Freeze-drying of serum albumin onto the surface of allografts reversed the tendency, thus the adhered BMSCs remained on the surface and showed moderate proliferation during the 18 day long experimental period (mean of pixels at the 3<sup>rd</sup> day:  $1658 \pm 278$ ; mean of pixels at the 18<sup>th</sup> day:  $2082, \pm 110$ ;  $p < 0.05$ ). Interestingly, the freeze-drying of fibronectin and collagen onto the surface of allografts did not affect positively either the initial adherence or the proliferation BMSCs compared to allografts coated with the aqueous form that of proteins (Figure 5).



**Figure 5. Adherence and proliferation of BMSCs on the surface of bone allografts coated with freeze-dried proteins.** The bar diagram shows that freeze-drying of albumin onto the surface of bone allografts significantly increased the quantity of initially adhered cells at day 3 compared to freeze-dried collagen or fibronectin coating. However, the multiplication of the adhered cells was moderate on the surface of freeze-dried albumin coated allografts during the experimental period. Panel A-B show representative images of freeze-dried albumin coated bone. The proliferation of the attached cells was observed on the day 18.

Dynamic seeding significantly increased the quantity of initially adhered BMSCs on the surface of the freeze-dried albumin coated allografts (Figure 6) compared to the standard culture conditions and the adhered cells showed intense proliferation (mean of pixels under standard condition at day 1:  $197 \pm 23$ , under dynamic conditions at day 1:  $9825 \pm 1208$ ; at the 7th day:  $15025 \pm 1704$ ). The same tendency was observed when DPSCs were seeded on the surface of freeze-dried albumin coated allografts under dynamic and standard culture conditions (standard condition: mean of reduced Alamar Blue (%) at 3<sup>rd</sup> day:  $14.5 \pm 2.23$ ; mean at 7<sup>th</sup> day:  $33.7 \pm 0.06$ ; dynamic condition: mean of reduced Alamar Blue (%) at 1st day:  $33.5 \pm 2.23$ ; mean at 7<sup>th</sup> day:  $60.9 \pm 1.09$ ).

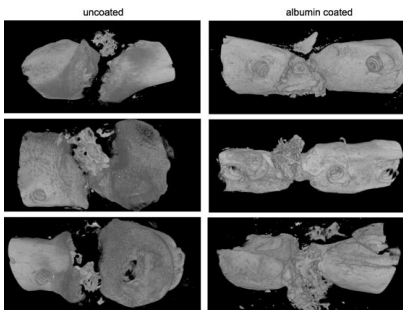


**Figure 6. Attachment of BMSCs and DPSCs onto the surface of freeze-dried albumin coated allografts under dynamic conditions.** The dynamic cell culture conditions markedly improved the initial attachment ( $p^{\dagger} < 0.05$ ) of both BMSCs (A) and DPSCs (B) which retained their capability of proliferating ( $p^{*} < 0.05$ ).



### 4.3. Osseointegration of freeze-dried human serum albumin coated human bone graft

Implantation of albumin coated bone grafts into a segmental bone defect model of delayed bone healing resulted in better integration of the freeze-dried albumin coated grafts than the uncoated ones. The implanted grafts were located in the defect in each case, but only the freeze-dried albumin-coated grafts triggered the ingrowth of new bone from the bone ends resulting in the union of the defect (Figure 7).



**Figure 7. Reconstructed 3-dimensional  $\mu$ CT images of osteotomized rat femurs after 4 weeks of the grafting procedure.** The left column shows that without freeze-dried albumin coating the allogeneic human bone graft does not coalesce with the bone ends and there is no bony consolidation. In contrast, when the grafts were coated with albumin there was apparent bone coalescence and a bony callus was formed.

## 5. Discussion

The results show that the *in vivo* biocompatibility of the chemically sterilized, antigen extracted freeze-dried human bone grafts is inferior to the comparator synthetic bone substitutes. The results supported that human serum albumin is a suitable coating substance to enhance the biological performance that of human bone grafts in *in vitro* and *in vivo* experimental settings. The freeze-drying procedure supports the reproducible biological performance of albumin coating, however, the homogeneity of albumin flakes is poor between the trabeculae of the human bone graft. The albumin coating does not influence the microhardness of the freeze-dried allografts;

the agitation under dynamic culture condition does not reduce its biological value. Intriguingly, the albumin coating increases mainly the initial adherence of MSCs on the surface of allografts, whereas significant proliferation is only seen after seeding under dynamic culture conditions. After implantation into a nonunion site, albumin coating improved the ingrowth of new bone from the host and resulted in the union of the bone ends. Interestingly, the albumin coating does not improve the *in vitro* biocompatibility of either the lyophilized bovine bone or the synthetic hydroxyapatite bone scaffolds.

There might be common reasons behind the low incorporation rate and the low biocompatibility with MSCs of freeze-dried human bone allograft. The chemical treatment of allografts kills not only pathogenic microorganisms and reduce the quantity of antigens but destroys the osteoinductive and osteogenic molecules turning bone grafts into mineralized scaffolds with reduced biological value. Although the mechanism of action of albumin coating is not known, but we hypothesize that it may play a crucial role in the recruitment and activation of osteogenic cells based on the volume expansion (colloidal suspension) theory.

After bone replacement surgery, alike after injury when bony injury occurs, rapid and active inflammatory response floods the injury zone with blood cells, platelets, monocytes, macrophages and other cells of the inflammatory cascade. The result of this process is that the injury site gets isolated from the rest of the body, becoming avascular to insure that the local injury environment does not propagate to the rest of the body. These segregation processes that occur at the bone fracture or injury sites eventually result in the repair blastema and outer surrounding reparative callus. The fracture hematoma has been proven to be a source of signalling molecules, such as interleukins, tumour necrosis factor- $\alpha$ , fibroblast growth

factor, insulin-like growth factor, platelet-derived growth factor, vascular endothelial growth factor, and the transforming growth factor  $\beta$  superfamily members that are supposed to induce a cascade of cellular events that initiate healing. These signal molecules might be adsorbed by the colloidal suspension of albumin that increases the local concentration of such biological cues, while it provides a natural delivery system that allows the prolonged release of those signals. This assumption is based upon the high, non-selective affinity of serum albumin to bind various biomolecules creating a high capacity natural buffer (reservoir) for them. The increased local concentration and sustained availability of those soluble cues may enhance cellular events in the repair tissue allowing the union of the bone ends in our animal model when the self-healing ability of the bone was compromised.

The same volume expansion theory of albumin may explain the *in vitro* performance of freeze-dried albumin coating. It seems to be a possible explanation that freeze-dried albumin adsorbs water when the cells are seeded onto the surface of allografts in aqueous media. The water adsorption may trigger the volume expansion of albumin yielding colloidal suspension that is temporally trapped in the channels of the allograft due to its high viscosity. This colloidal suspension of albumin may embed MSCs and keep them in the pores and channels of the allograft providing enough time to produce extracellular matrix and establish focal adhesions with the surface.

## 6. Conclusions

**6.1.** Chemically sterilized, antigen-extracted freeze-dried human bone grafts showed reduced *in vivo* osseointegration in compromised bone defect model compared to synthetic bone fillers.

**6.2.** Freeze-dried human serum albumin is a suitable substance for the reproducible coating of cancellous human bone grafts.

**6.3.** The freeze-dried human serum albumin coating improved the *in vitro* and *in vivo* biocompatibility of the chemically sterilized, antigen-extracted human bone grafts.

## 7. Publications

### Publications related to the present thesis

[1] **Weszl M**, Skaliczki G, Cselenyák A, Kiss L, Major T, Schandl K, Bognár E, Stadler G, Peterbauer A, Csöngé L, Lacza Z. Freeze-dried human serum albumin improves the adherence and proliferation of mesenchymal stem cells on mineralized human bone allografts. *J Orthop Res.* 2012 Mar;30(3):489-96.

[2] Hulsart-Billström G, Xia W, Pankotai E, **Weszl M**, Carlsson E, Forster-Horváth C, Larsson S, Engqvist H, Lacza Z. Osteogenic potential of Sr-doped calcium phosphate hollow spheres *in vitro* and *in vivo*. *J Biomed Mater Res A.* 2013 Aug;101(8):2322-31.

[3] Aberg J, Pankotai E, Hulsart Billström G, **Weszl M**, Larsson S, Forster-Horváth C, Lacza Z, Engqvist H. *In vivo* evaluation of an injectable premixed radiopaque calcium phosphate cement. *Int J Biomater.* 2011;2011:232574.

### Publications not related to the present thesis

[1] Horváthy DB, Váczi G, Cselenyák A, **Weszl M**, Kiss L, Lacza Z. Albumin-coated bioactive suture for cell transplantation. *Surg Innov.* 2013 Jun;20(3):249-55.

[2] Skaliczki G, **Weszl M**, Schandl K, Major T, Kovács M, Skaliczki J, Redl H, Szendrői M, Szigeti K, Máté D, Dobó-Nagy C, Lacza Z. Compromised bone healing following spacer removal in a rat femoral defect model. *Acta Physiol Hung.* 2012 Jun;99(2):223-32.

[3] Skaliczki G, Schandl K, **Weszl M**, Major T, Kovács M, Skaliczki J, Szendrői M, Dobó-Nagy C, Lacza Z. Serum albumin enhances bone healing in a nonunion femoral defect model in rats: a computer tomography micromorphometry study. *Int Orthop.* 2013 Apr;37(4):741-5.

[4] Terdik A, Klára T, Csöngé L, Lacza Z, Bognár E, **Weszl M**. Csontpótló anyagok összehasonlító mikrokeménység vizsgálata *BIOMECHANICA HUNGARICA* 6:(2) pp. 13-17. (2013)