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Interaction of Sheep Bone Marrow Stromal Cells With Biodegradable Polyurethane Bone Substitutes†

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Summary: Autogenous cancellous bone graft is often used in clinical practice to promote bone healing in large, critical-size defects. Bone harvesting has numerous drawbacks which calls for new alternatives. Potentially, an alternative to bone grafting may be offered by a tissue engineering approach that utilizes a suitable scaffold seeded with osteogenic cells. In this study the interaction of sheep bone marrow stromal cells with biodegradable microporous polyurethane scaffolds designed as bone substitutes was investigated. The scaffolds were produced from the same polyurethane but had two different pore sizes in the ranges of 140–400 μm and 200–600 μm. The scaffolds were impregnated with platelet-rich plasma or fibrin to evaluate whether impregnation affects cell attachment, growth and proliferation. It has been found that scaffolds promote attachment, differentiation and proliferation of sheep bone marrow stromal cells. The cells adhered well to the scaffold's surface and infiltrated its porous structure. The DNA content and the mRNA expression increased with time of culture. Histological and immunohistochemical stainings showed that the cells were viable and differentiated into osteoblasts. There was an evident effect of the scaffolds' pore size on cell attachment and proliferation. Cell growth was more efficient in the scaffolds with smaller pore sizes than in the scaffolds with larger pores. Scaffolds with larger pores underwent calcification. No calcium was found in the scaffolds with smaller pore sizes. The pore size had no effect on cell morphology. The stromal cells differentiated into osteoblasts, which process was more pronounced in the scaffolds with larger pores. Impregnation of the scaffolds with fibrin or platelet-rich plasma facilitated cell growth and proliferation. This effect was more pronounced for scaffolds impregnated with platelet-rich plasma than for scaffolds impregnated with fibrin.

Keywords: biodegradable, bone substitutes, cell culture, Polyurethane scaffold, stem cells, tissue engineering

Introduction

Large, critical-size bone defects which are defined as ''defects which do not heal spontaneously during the life time of the animal''[1] and/or defects which show less than 10 percent of bony regeneration during the lifetime of the animal''[2], usually require bone grafting to promote healing. Harvesting of cancellous bone graft from

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the ilium which is the ''gold standard'' has a number of drawbacks. Long healing time of monocortical defects, no healing of bicortical and tricortical defects and high morbidity of the donor site are examples.[3–5]

The risk of infections with HIV or hepatitis and possible immune reactions are the main concerns with the use of allografts or xenografts.[6–7] Bone substitutes based on calcium phosphate ceramics also have their limitations.[8] Hence, there are continuing efforts to develop new, more functional bone substitutes.

An ideal bone substitute should fulfil a number of requirements. Thus, it should be osteoconductive, osteogenic and osteoinductive to support attachment and growth of osteogenic cells and the production of extracellular matrix. It should be porous with interconnected pores to allow for the ingrowth of cells and newly formed bone. Preferably it should be bioresorbable and/or biodegradable to allow replacement with bone. Radiolucency of such substitute would be an asset as bone healing could be followed up by simple X-ray technique.[9] Loading the substitute with autogenous bone marrow, platelet-rich plasma (PRP) or seeding it with osteogenic cells would provide osteoinductive properties, while incorporating calcium phosphate ceramics would enhance osteoconductivity.

Among candidate materials for such bone substitutes are biodegradable polymers, and particularly polyhydroxyacids and new biodegradable polyurethanes. The later materials are of special interest as their degradation rates, elastic properties and hydrophilicity can be controlled during synthesis to promote interaction with cells and tissues.[10–17] In addition, their biological properties such as osteoconductivity and osteoinductivity can be promoted by impregnation with autogenous bone marrow, platelet-rich plasma and/or by seeding with osteogenic cells.

This study addresses the ability of new biodegradable porous polyurethane scaffolds designed for cancellous bone graft substitution to support attachment, growth and proliferation of sheep bone marrow stromal cells. The scaffolds seeded with osteogenic cells might potentially be used as a replacement for cancellous bone graft.

Materials and methods

Polyurethane Scaffolds

The details on the preparation of the isosorbide-based biodegradable polyurethane scaffolds used in the study have been given elsewhere.[16] The scaffold blocks were cut into discs 8×4 mm, packed in double pouches, sterilized using a cold-cycle ethylene oxide process and subsequently evacuated at 50 °C and 3×10^{-1} mbar for 48 hours. The as-produced scaffolds were used as a control. In addition, the as-produced scaffolds were also impregnated with fibrin or platelet-rich plasma.

Bone Marrow Stromal Cells and Blood

Bone marrow (30 ml) and the blood aspirates (50 ml) were harvested from sheep undergoing surgery and collected in EDTA-containing Sarstedt monovettes®. The samples stored at room temperature were processed within 6 hours after harvesting.

Preparation of Platelet-Rich Plasma

The blood aspirates were transferred from Sarstedt monovettes® into 15 ml Falcon tubes and centrifuged at room temperature for 30 min at 200 g. The resulting plasma supernatants were pooled, transferred into new 15 ml Falcon tubes and centrifuged at room temperature at 200 g for 5 min to get pellets containing platelets. Platelet-rich plasma (PRP) obtained by resuspending the pellets in 1 ml of the remaining plasma supernatant was stored at −20 °C.

Isolation and Expansion of Cells

Bone marrow aspirates were homogenized and centrifuged at room temperature (5 min, 200 g) and the top monolayer containing blood and fat was disposed of. The remaining pellets containing cells were resuspended in IMDM culture medium with 10% fetal bovine serum (FBS, Gibco

10270-106) and 0.01% bFGF (R&D 233-FB) and injected into 300 cm^3 T-flasks. The isolated cells were cultured using IMDM culture medium, 10% FCS and 0.01% bFGF at 37 °C by 5% CO_2 and 95% humidity. Initially, the monolayers were washed every day with Tyrode's balanced salt solution (TBSS) to remove the remaining blood cells and nonadherent cells. The medium was changed three times a week and cells were subcultured 1:3 at subconfluence. Adherent cells were termed mesenchymal stem cells (MSC) after one subculture. Cells at passage 4 were used for the experiment only.

Cell Culture

The scaffolds after sterilization were degassed in IMDM medium for 4 hours under vacuum. The mesenchymal stem cells (MSC) were trypsinized, washed twice and after each washing centrifuged at room temperature for 10 min at 380g. The resulting pellet was resuspended in IMDM medium containing 10% FBS. The cells were stained with methylene blue, counted using a homocytometer, centrifuged at 380g for 10 minutes and resuspended in PRP or fibrin sealant (Baxter Biosurgery, Vienna, Austria), at a concentration of 4×10^6 cells/150 μl. The cell/fibrin or cell/PRP suspensions before loading into the scaffolds were activated by addition of 15 μl of bovine thrombin dissolved in PBS (Sigma T-4648, final concentration 5 U/ml). The cell/fibrin or cell/PRP suspension was pipetted into sterile Eppendorf tube caps. The polyurethane scaffolds were squeezed using forceps and placed into the caps. Release of pressure applied to the scaffolds resulted in sucking the cell suspension into the scaffolds. Subsequently, the scaffolds were turned upside down and the cells were uniformly distributed in the scaffolds due to gravitational force. The scaffolds in the caps were incubated for 30 min at 37 °C, transferred to six-well polystyrene plates and covered with IMDM culture medium containing 10% FCS, 1% pentomycin-streptomycin (100 U/ml, Gibco 15140-122), 100 μM L-ascorbic acid 2-phosphate (Gibco 11140-035) and 10 nM of dexamethasone (Sigma D-2915). The wells were placed in an incubator at 37 °C, 95% relative humidity, 5% CO_2. The medium was changed three times a week. Each time the medium was changed, the polyurethane scaffolds with cells were transferred to new 6-well polystyrene wells and covered with the IMDM culture medium containing 10% FCS, 1% pentomycin-streptomycin (100 U/mL, Gibco 15140-122), 100 μM L-ascorbic acid 2-phosphate (Gibco 11140-035) and 10 nM dexamethasone (Sigma D-2915). The cells were cultured for 2, 10, 20 and 30 days.

DNA Quantification

The scaffolds with cells were placed in Eppendorf tubes and the cells were digested at 56 °C overnight by adding 1 ml proteinase K (0.5 mg/ml proteinase K in phosphate buffer containing 3.36 mg/ml disodium-EDTA solution). After dilution with Dulbecco's phosphate buffered saline (DPBS) containing 0.1% v/v of H33258 from 1 mg/ml stock (Polysciences Inc, Warrington, PA, USA), DNA quantification was carried out according to the procedure described elsewhere (PE HTS 7000 Bio Assay Reader, 360 nm excitation, 465 nm emission wavelength).[15]

RNA Isolation and Reverse Transcription

The scaffolds at each time point were placed in Eppendorf tubes and pulverized. The mRNA was extracted using 1 ml of TRI-Reagent (TR-118, Molecular Research Center, Inc, Cincinnati, OH, USA). Next 150 ml of 1-bromo-3-chloro-propane was added to the tubes, the tubes were vortexed for 15 sec and centrifuged at 12000 g for 15min at 4 °C. The colourless top layer was transferred into new tubes, 750 μl of isopropanol was added and the tubes were centrifuged at 12,000 g for 10 min at 4 °C. Sample supernatants were removed and pellets were washed in 1 ml of 75% ethanol by vortexing and centrifuging at 10,000 g for 5 min at 4 °C. Pellets were air-dried and resuspended in 30 μl of diethyl pyrocarbonate - water solution (DEPC). The tubes were kept at 60 °C for

15 min and then placed in ice. The total RNA amount and purity was assessed by measuring the absorbance at 260 and 280 nm. The TaqMan Reverse Transcription Reagents kit (Perkin Elmer No. N808-234) was used to determine the reverse transcription using 1 μg of total RNA sample and DEPC was added to bring the final reaction volume to 20 μl. The measurements were carried out using a Thermal Cycler 9600 instrument (Applied Biosystems, DriveFoster City, CA, USA). The cDNA samples were diluted to 250 μl with Tris-EDTA buffer (SIGMA T-9285) before being used for real-time RT PCR.[16]

Real-time PCR

Oligonucleotide primers and TaqMan probes were purchased from Applied Biosystems (Assay-On-Demand, Applied Biosystems, DriveFoster City, CA, USA). Primers and probes for amplification of 18S ribosomal RNA, used as endogenous controls, were all from Applied Biosystems. All other primers and labeled TaqMan probes were from Microsynth (Balgach, CH). TaqMan probes were labeled with the reporter dye molecule FAM (6-carboxyfluorescein) at the 5′end and with the quencher dye TAMRA (6-carboxy-N, N, N′, N′-tetramethylrhodamine) at the 3′end. The different primers Collagen type 1, MMP-13, and ADAMTS 4 for bone development were chosen for analysis. Assays-On-Demand oligonucleotides were mixed according to the manufacturer's protocol. The PCR reaction mixture contained TaqMan Universal PCR master mix without AmpErase UNG (Applied Biosystems, cat# 4324018), 900 nM primers (forward and reverse), 250 nM TaqMan probe, and 2 μl of cDNA sample for a total reaction volume of 25 μl. PCR conditions were 95 °C for 10 min, followed by 42 cycles of amplification at 95 °C for 15 sec and 60 °C for 1min using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative quantification of mRNA targets was performed according to the comparative CT method with 18S ribosomal RNA as the endogenous control (ABI PRISM 7700 Sequence Detector User Bulletin 2, PE Applied Biosystems, 1997).

Histology

The scaffolds were fixed in pure methanol for one week at 4 °C and then kept in 5% sucrose in PBS for 24 hours before sectioning. Subsequently, the samples were embedded in Microm embedding medium (Walldorf, Switzerland, cat no. 350100), and the slices with a thickness of 10 μm were cryosectioned using a Cryostat-Microtome HM 560 OMV (Carl Zeiss AG, Zürich, CH). The samples were evaluated under Super-Frost Plus GOLD microscope (Menzel-Gläser, Braunschweig, Germany). Another set of slices was frozen at −20 °C, then dried at room temperature for at least 1 week and stained with toluidene blue (1% toluidene blue, Fluka, Switzerland, cat. no. 89640 in aqueous solution of 1 g sodium tetraborate, Fluka, cat no. 71998 in double-deionized water) and then rinsed twice in deionized water. Van Kossa staining was carried out by incubating the slices for 30 minutes in 5% aqueous solution of silver nitrate (Fluka cat no. 85230), followed by exposure to UV light, rinsing with deionized water, incubating for 10 minutes in 5% aqueous solution of sodium thiosulfate (Fluka No. 72050), rinsing with deionized water and counterstaining with Nuclear Fast Red (Fluka No. 60700) followed by rinsing with deionized water. The dried slices were stained for 5 minutes in Mayer's haematoxylin, moved into lukewarm tap water for at least 5 minutes and then rinsed in double-deionized water. Next, the samples were stained for 3 minutes in eosin (Fluka No. 45240) and rinsed again in double-deionized water. During all staining procedures the coverslips were mounted using an aqueous mounting solution (Hydromount HS-106, National Diagnostics, Atlanta, GA, USA).

Immunofluorescence

The dried sections were encircled using a hydrophobic Dako Cytomation Pen (Dako-Cytiomation, DK-2600 Glostrup, Denmark A/S) and were left to rehydrate in PBS-Tween (0.32 g/l NaH_2PO_4, 1.42 g/l Na_2HPO_4,

9 g/l NaCl, 0.1% Tween-20, pH 7.4) for 5 min. Non-specific binding was reduced by incubating the samples for 60 min at room temperature in horse serum (diluted 1:20 in PBS, by Vector Labs, USA). The primary antibody (polyclonal rabbit anti-human OSF-2 by BioVendor cat. no. RD181045050) was added in the appropriate dilution (OSF-2 1:400) and the samples were incubated for 3 hours at room temperature. PBS-Tween was used as a control. The samples were then washed 3 times with PBS-Tween before being incubated with the fluorescent secondary antibody (OSF-2: Alexa Fluor 488 goat anti-rabbit IgG antibody at 1:400 dilution by Molecular Probes cat. no. A11008) for 30 minutes at room temperature. Next, the samples were washed, mounted on coverslips using ProLong Gold Antifade reagent (Molecular Probes cat# P36930) and stored in the dark until analysis.

Microscopical Analysis

All histological sections were analyzed using a Zeiss Axioplan 2 Microscope, a Zeiss Axiocam camera and a Zeiss Axiovision software.

Statistics

The results are expressed as mean $\pm$ SEM of triplicate samples of independent experiments. The statistical analysis was done using the non-parametric Mann-Whitney U-test, which compares the medians of two independent distributions. $P < 0.05$ was considered to be statistically significant.

Results

Polyurethane Scaffolds

The scaffolds used in cell culture experiments had two different pore sizes. The pore size of the scaffolds marked as SP039 was in the range of 140–400 μm, and the pore size of the scaffolds marked as SP040 was in the range of 200–600 μm (Figure 1).

DNA Expression

The values of the DNA expression measured at four different time points (day 2, 10, 20, and 30) indicated a constant growth of cells in all scaffolds. In the scaffolds impregnated with PRP the cell growth was more efficient than in the scaffolds impregnated with fibrin. The best results were obtained for the scaffold SPO39 with pore sizes in the range of 140 to 400 μm impregnated with PRP (Figure 2).

Gene Expression

RT-PCR measurements of three different osteoblastic gene expressions at the four different time points were carried out to evaluate the efficiency of attachment and proliferation of mesenchymal stem cells to the polyurethane scaffolds. The genes evaluated were collagen type 1, MMP-13 and ADAMT-4. It has been found that the gene expression levels of collagen type 1 and MMP-13 at all time points were always higher for the scaffold SPO40 with the larger pore sizes than for the scaffolds SPO39 with smaller pore sizes. Irrespective of the scaffold's pore size, however,

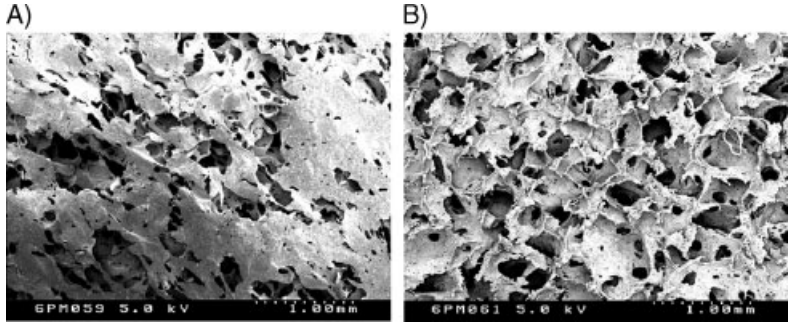

Figure 1.
A. SEM image of porous structure of PU scaffold SPO39; B. SEM image of porous structure of PU scaffold SPO40.

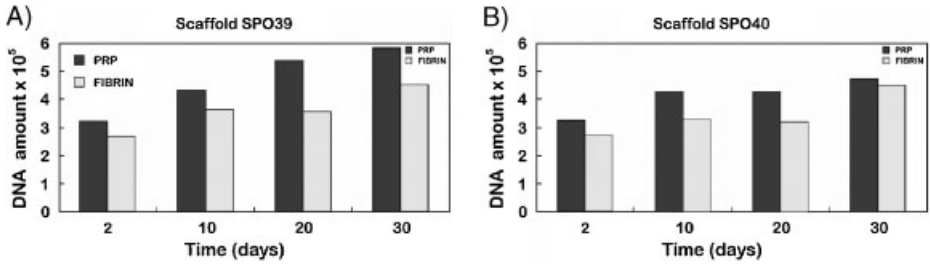

Figure 2.
A. DNA amount in SPO39; B. DNA amount in SPO40.

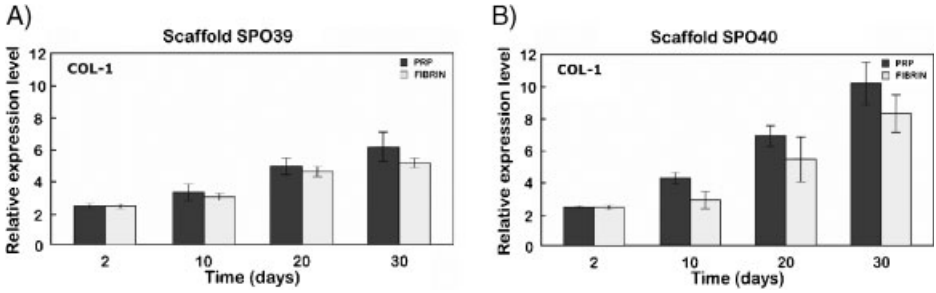

Figure 3.
Gene expression levels measured by real-time PCR: A. RNA expression of COL-1 in SPO39; B. RNA expression of COL-1 in SPO40.

attachment and proliferation of mesenchymal stem cells was always more efficient in the scaffolds impregnated with PRP than in the scaffolds impregnated with fibrin (Figure 3).

The MMP-13 gene expression was similar to collagen type 1 gene expression at all four time points. In all the cases, the gene expression increased with time of cell culture, and for the scaffolds with the same pore size it was higher for the scaffolds impregnated with platelet-rich plasma than for the scaffolds impregnated with fibrin (Figure 4).

Similar results for gene expression were also obtained for ADAMTS-4 for all 4 time

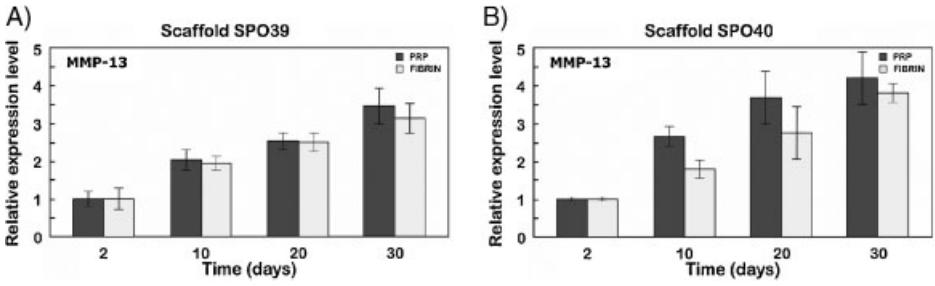

Figure 4.
Gene expression levels measured by real-time PCR: A. RNA expression of MMP-13 in SPO39; B. RNA expression of MMP-13 in SPO40.

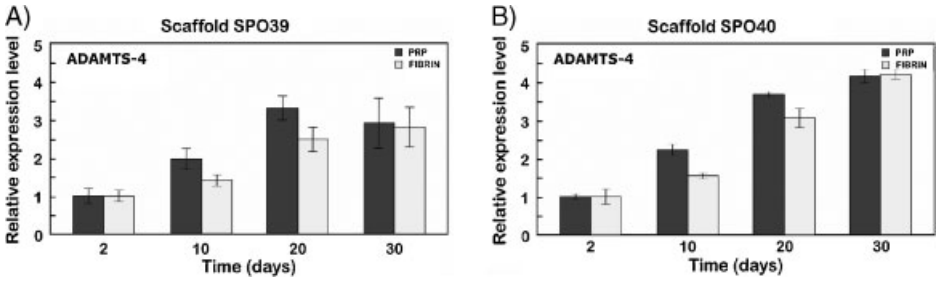

Figure 5.
Gene expression levels measured by real-time PCR: A. RNA expression of ADAMTS-4 in SPO39; B. RNA expression of ADAMTS-4 in SPO40.

points (Figure 5). The differences in COL-1 or MMP-13 for the scaffolds impregnated with fibrin or platelet-rich plasma were even more evident at day 30 of the cell seeding experiments. For the SPO39 scaffold impregnated with platelet-rich plasma there was a down-regulation in gene expression at day 30. In consequence, the results for gene expression were similar irrespective of whether the scaffolds were impregnated with platelet-rich plasma or fibrin.

Histological Analysis

The polyurethane scaffolds impregnated with fibrin or platelet-rich plasma and seeded with cells were stained with haematoxylin-eosin at the time point 1 (day 2) to control whether there was a uniform distribution of cells throughout the whole scaffold. The results are shown in Figure 6 (scaffold SPO39) and in Figure 7 (scaffold SPO40). It can be seen from the pictures that the distribution of cells in the scaffolds was uniform.

The scaffolds seeded with cells at time point 4 (day 30) were stained with toluidine blue to evaluate whether cells in the scaffolds changed shape. It has been found that there was no change in cell morphology, irrespective of whether the scaffolds were impregnated with fibrin or platelet-rich plasma. The scaffolds' pore size also had no effect on cells morphology (Figure 8 and 9).

Van Kossa staining showed that only SPO40 scaffolds with larger pores impregnated with fibrin or platelet-rich plasma underwent calcification, the effect being more pronounced for the scaffolds impregnated with platelet-rich plasma than for the scaffolds impregnated with fibrin (Figure 10).

As has been stated above, the SPO39 scaffolds with smaller pore size did not calcify irrespective whether they were

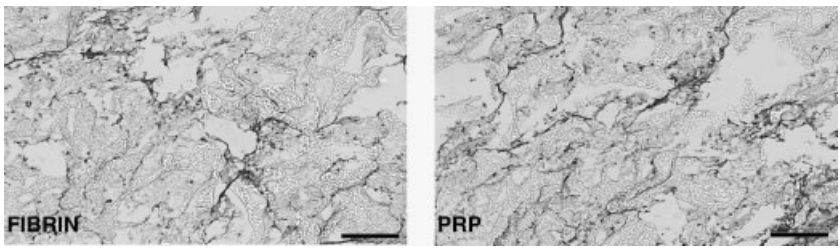

Figure 6.
Haematoxylin-Eosin staining. SPO39 seeded with fibrin and PRP at day 2: Equal distribution of bone marrow stromal cells in the scaffolds. (bar represents 200 μm)

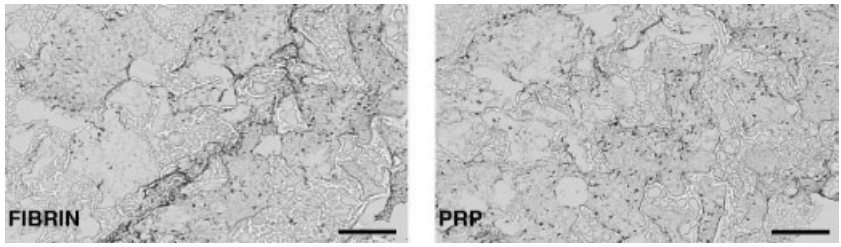

Figure 7.
Haematoxylin-Eosin staining. SPO40 seeded with fibrin and PRP at day 2: Equal distribution of bone marrow stromal cells in the scaffolds. (bar represents 200 μm)

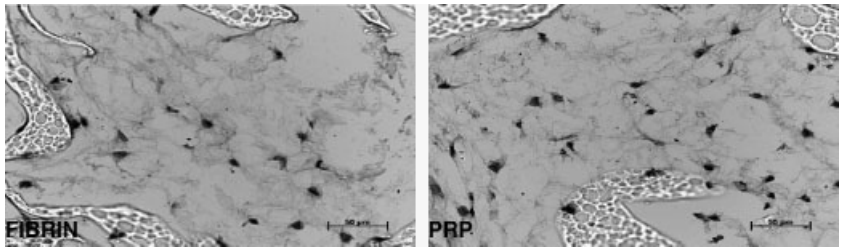

Figure 8.
Toluidine Blue at day 30. Scaffolds SPO39 seeded with fibrin, SPO39 seeded with PRP: The constructs did not show remarkable morphological characteristics. The scaffold SPO39 seeded with PRP showed the most cells. (bar represents 50 μm)

impregnated with fibrin or platelet-rich plasma. The immunofluorescence analysis using an antibody against the osteogenic transcription factor OSF-2 showed that the stem cells seeded in the scaffolds differentiated into osteoblasts. The number of osteoblastic cells was higher in the SPO40 scaffolds than in the SPO39 scaffolds. The number of differentiated cells was higher in the scaffolds impregnated with platelet-rich plasma than in the scaffolds impregnated with fibrin (Figure 11).

Discussion

The loss of tissues or internal organs is frequent. Transplantation is often the only

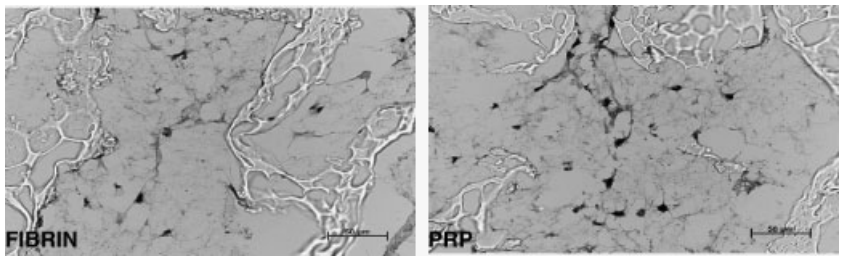

Figure 9.
Toluidine Blue at day 30. Scaffolds SPO40 seeded with fibrin, SPO40 seeded with PRP: The constructs did not show remarkable morphological characteristics. (bar represents 50 μm)

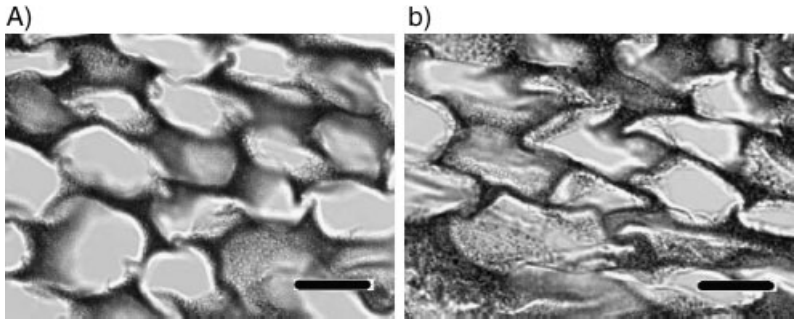

Figure 10.
van Kossa staining. A. Calcification in multiple areas of SPO40 seeded with fibrin; B. Calcification in multiple areas of SPO40 seeded with PRP. (bar represents 200 μm)

means to restore body functions, but availability of spare tissues for transplantation is limited. This calls for new treatment modalities which would utilize alternatives to autogenous tissues. Potentially, an alternative may offer the tissue engineering approach utilizing constructs based on suitable scaffolds seeded with autogenous cells. Depending on the tissues to be repaired and the cells used, the scaffolds may vary in respect to pore size, structure and interconnectivity, mechanical properties and, importantly, on the chemical and physical characteristics of the surface, a factor that is especially critical in the first stages of cell attachment.

Among various candidate materials for scaffolds are bioresorbable/biodegradable polymers. In this group biodegradable polyurethanes are of special interest as the versatile chemistry of these materials allows tuning their properties in accordance with the required application [Gorna, Gogolewski]. Although ideally, the scaffolds in their original form should facilitate cell attachment and proliferation, ''post-treatment'' often the scaffolds' often has to be carried out to enhance their interaction with cells. One of the most common methods to promote cell-material interaction is to ''biolize'' scaffolds with proteins, primarily, with fibrin and/or platelet-rich plasma. This treatment also temporarily reduces the scaffold's pore size and prevents the cells loaded into the scaffold and the extracellular matrix from being washed out. In addition, platelet-rich plasma which carries growth factors and cytokines up-regulates osteoblastic genes, thus promoting cell growth and proliferation.

In the present study sheep mesenchymal stem cells were seeded into the biodegradable polyurethane scaffolds designed as cancellous bone graft substitutes to assess

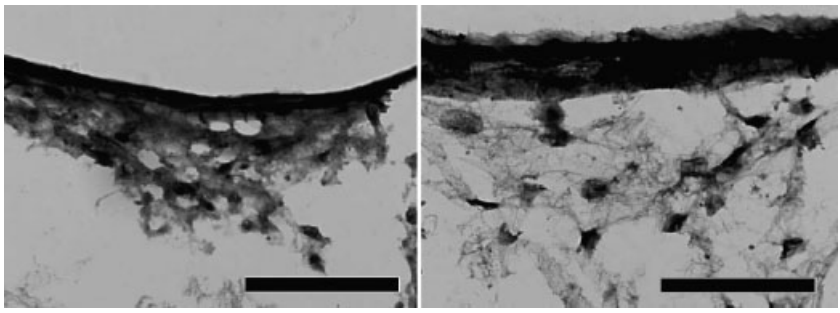

Figure 11.
OSF-2 staining. Differentiation of SPO40 with fibrin. Differentiation of SPO40 with PRP.

their potential to support cell attachment and proliferation. The scaffolds varied in pore size and were produced from the new experimental biodegradable polyurethane based on biologically active isosorbide diol [Gorna, Gogolewski]. The scaffolds were ''biolized'' by impregnation with fibrin or platelet-rich plasma, the procedures used clinically to promote bone healing, and seeded with bone marrow stromal cells to evaluate whether this treatment promotes attachment and growth of cells. In both cases the biolization of the scaffolds facilitated cell attachment and proliferation although as might be expected, the impregnation with platelet-rich plasma promoted this process more effectively.

Conclusions

Biodegradable isosorbide-based porous polyurethane scaffolds promote attachment, growth and proliferation of bone marrow stromal cells in culture and their differentiation into osteoblasts. The scaffold's pore size plays an important role in this process, i.e. there was a higher number of cells in the scaffolds with smaller pores than in the scaffolds with larger pores. The pore size also played a role in scaffold calcification. Thus, only the scaffolds with larger pores nucleated the deposition of calcium phosphate salts. No calcium was found in the scaffolds with smaller pores. Impregnation of the scaffolds with fibrin or platelet-rich plasma facilitates cell growth and proliferation, the effect being more pronounced for scaffolds impregnated with platelet-rich plasma than for scaffolds impregnated with fibrin. The biodegradable polyurethane scaffolds seeded with osteogenic cells may potentially be used as substitutes for autogenous cancellous bone graft in the treatment of large bony defects. The presence of autogenous osteogenic cells in the scaffold may promote bone healing.

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