2

Autologous Cell Therapies for Bone Tissue Regeneration

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1. Introduction

Bone tissue engineering using various cell sources and materials has become an intriguing field, aimed at solving the problem of treatment of numerous clinical indications requiring regeneration of damaged or deficitary bone.

During the Paleozoic period, evolution produced the skeleton. This 500 million-year-old creation has the capacity for regeneration, a term which until recently has been reserved to new tissue and organs formation as in hydra, planarians or salamanders (Braddock *et al.*, 2001). The regeneration of bone (or the stimulation of bone production) is often required to treat loss of bone tissue brought about by trauma, osteonecrosis and tumors. Among the 6 millions fractures occurring every year in the United States, 5-10% are classified unfavorable, requiring further treatment due to compromised healing (Praemer et al., 1992). The clinical and socioeconomic challenge of treatments of bone defects is staggering. For example, the number of total joint arthroplasties (TJAs) and revision surgeries in the US has increased from 700,000 in 1998 to over 1.1 million in 2005. Medical expenses relating to fracture, reattachment, and replacement of hip and knee joint was estimated to be over $20 billion (USD) in 2003, and predicted to increase to over $74 (USD) billion by the year 2015. Similar trend is observed in spinal arthrodesis (reviewed in Porter et al, 2009).

Bone tissue provides mechanical stability to the skeleton, which is needed for load bearing, locomotion and protection of internal organs. Furthermore, bone serves as a mineral reservoir and has the capacity rapidly to mobilize mineral stores if needed for homeostasis of the calcium blood level (Kneser *et al.*, 2006).

The functional integrity of bone tissue is maintained by three main different cell types: osteoblasts, osteocytes and osteoclasts, which are embedded in a highly complex matrix consisting of a mineralized (hydroxyapatite) and a non-mineralized component. The non-

mineralized organic part contains mainly collagens (approx. 95%), the remaining organic component of 5% is composed of glycoproteins, proteoglycans and other numerous non-collagenous proteins (Meyer and Wiesmann, 2006).

Bone development and bone regeneration are complexly regulated processes that involve a plethora of different growth and transcription factors, which coordinate the interaction of cells and matrix in response to external or internal stimuli (Kneser *et al.*, 2006). Bone metabolism involves the resorption of existing bone by osteoclasts and the subsequent formation of a new bone matrix by osteoblasts. These activities are essential for bone remodeling, regeneration and repair (DeLong *et al.*, 2007).

Various sources of cells (periosteal cells, cortical cells, cells derived from the surrounding soft tissues, and marrow cells) and signals that set up these fields are responsible for the features of the repair tissue. The primary tissue source of cells that form repair tissue is believed to be from the periosteum. Other cells that contribute to or repair tissue formation appear to be derived from the adjacent cortical and cancellous bone. Mesenchymal stem cells, assumed to be derived from either the surrounding muscle tissue or the marrow space, are a third source of cells that participate in the formation of new bone. Cells synthesize a network of collagnenous and non-collagenous proteins. The final stage of bone repair and regeneration is the establishment of mineralized, mechanically competent tissue. Collagens, as the major constituent of the extracellular matrix network, are of major importance in the formation of a mineralized matrix. (Nakahara *et al.*, 1990; Meyer and Wiesmann, 2006)

The healing potential of bone is sufficient to restore simple fractures, which are generally treated by standard conservative or surgical therapy. However, in some cases, reparative osteogenesis does not result in structural and functional recovery of the bone (Logeart-Avramoglou *et al.*, 2005). Extended bone defects following trauma or cancer resection or non-unions of fractures may require more sophisticated treatment. In these cases, bone grafting procedures, segmental bone transport, distraction osteogenesis or biomaterials are applied for reconstruction (Meyer and Wiesmann, 2006). The repair of bone defects in reconstructive surgery is subject to significant limitations, including donor site morbidity, limited supply of autograft, risk of infection and immune rejection of allograft, and poor osteogenic effect of synthetic bone substitutes (Logeart-Avramoglou *et al.*, 2005). In addition, bridging of a large bone defect by callus distraction requires a long time and usually an external fixator, both very inconvenient for patients. Regardless of the technique used, the percentage of failure is considerable. Bone repair is therefore the subject of intensive investigation in reconstructive surgery.

2. Treatment of bone defects

The reconstruction of large bone defects is an important clinical problem and none of the approaches thus far have proved completely effective. Since there are major limitations when treating ”problematic” bone tissue defects according to standard protocols, there is a great need for the development of new approaches for reparative osteogenesis. There are a number of clinical indications, such as non-unions, benign bone lesions, parodontal bone lesions, traumatic injuries, which could benefit from advances made during the past decade in bone cell therapies and tissue engineering. Cell therapies involve the use of any kind of cells to repair damaged or destroyed bone cells or tissues, and are unique in that the active component consists of living cells.

One of the biggest cell therapy areas is tissue engineering, defined by Langer and Vacanti (1993) as an interdisciplinary field that applies the principles of engineering and the life sciences to the development of biological substitutes that restore, maintain or improve tissue function. Tissue can be engineered 1) *in vivo* - by stimulating the body's own regeneration response with the appropriate biomaterial, or 2) *ex vivo* - cells can be expanded in culture, attached to a scaffold and then reimplanted into the host. Depending on the source, cells may be heterologous (different species), allogeneic (same species, different individual) or autologous (same individual). Autologous cells are preferred because they will not evoke an immunologic response and the deleterious side effects of immunosuppressive agents can thus be avoided. In addition, the potential risks of pathogen transfer are also eliminated (Hipp and Atala, 2004).

When engineering bone tissue substitutes, mechanical stability, osteoconductivity, osteoinductivity, osteogenicity and ease of handling have to be well balanced in order properly to meet clinical needs (Kneser *et al.*, 2006). According to Muschler and co-workers (2004), there are four types of cell-based tissue engineering: (1) local targeting of connective tissue progenitors where new tissue is needed, (2) transplanting autogenous connective tissue progenitors to augment the local population, (3) transplanting culture expanded or modified connective tissue progenitors and (4) transplanting fully formed tissue.

Osteogenic cells are an integral part of any bone tissue engineering strategy. These cells are either transplanted along with the appropriate scaffolds into the bone defects or attracted from the host by osteoinductive factors (Kneser *et al.*, 2006). The affectors of bone remodeling, regeneration and fracture repair in an adult organism are the cellular components. Various types of osteogenic cells, including bone marrow mesenchymal stem cells (BMSC) (Frank *et al.*, 2002; Meinel *et al.*, 2004; Meinel *et al.*, 2005), adipose-derived stem cells (ASC) (Lendeckel *et al.*, 2004; Peterson *et al.*, 2005) mesenchymal cells of the periosteum (Hutmacher and Sittinger, 2003; Schimming and Schmelzeisen, 2004, Turhani *et al.*, 2005) and alveolar bone derived osteoblasts (AO) (Xiao *et al.*, 2003; Zhu *et al.*, 2006) have been studied for bone reconstruction. Pluripotent mesenchymal stem cells are present in many adult tissues, although they are most abundant in bone marrow (Pittenger *et al.*, 1999) and adipose tissue (Zuk *et al.*, 2001). *In vitro*, BMSC are rapidly adherent, clonogenic and capable of extended proliferation (Bianco *et al.*, 2001). Isolation and expansion efficiency, stability of osteoblastic phenotype, *in vivo* bone formation capacity and long-term safety are essential requirements that must be met by any type of osteogenic cell for successful clinical application. Serum-free culture conditions or culture medium supplemented with autologous serum are preferable for cell expansion *in vitro* (Kneser *et al.*, 2006).

Nevertheless, it has still not yet been determined which type of osteogenic cell is most suitable for engineering bone tissue. At the moment, BMSC seem to be the best candidate for cell therapy to regenerate injured skeletal tissues, owing to their ease of isolation, expansion and multilineage potential. These cells can be induced to differentiate into chondrocytes or osteoblasts when subjected to specific environmental factors (Jorgensen *et al.*, 2004). Proof-in-principle for bone tissue engineering using BMSC has been demonstrated in various animal models (for review see Cancedda *et al.*, 2003); in addition, 7 human clinical studies had been conducted by 2010 (Chatterjea *et al.*, 2010). However, several studies have also shown ASC and AO to be appropriate cell sources for bone regeneration (Zuk *et al.*, 2001; Cowan *et al.*, 2004; Hattori *et al.*, 2006; Fröhlich *et al.*, 2010; Turhani *et al.*, 2005; Maličev *et al.*, 2008).

Bone tissue engineering requires not only living cells but also the use of scaffolds, which serve as a three-dimensional environment for the cells. Scaffolds for engineering bone should satisfy a number of criteria. According to Logeart-Avramoglou *et al.* (2005), such matrices should be: (i) biocompatible (non-immunogenic and non-toxic); (ii) absorbable (with rates of resorption commensurate with those of bone formation); (iii) preferably radiolucent (to allow the new bone to be distinguished radiographically from the implant); (iv) osteoconductive; (v) easy to manufacture and sterilize; and (vi) easy to handle in the operating theater, preferably without preparatory procedures (in order to limit the risk of infection). Three-dimensional scaffolds for bone tissue regeneration require an internal microarchitecture, specifically highly porous interconnected structures and a large surface-to-volume ratio, to promote cell in-growth and cell distribution throughout the matrix (Logeart-Avramoglou *et al.*, 2005). Pore sizes in the range of 200-900 μm have performed most satisfactorily in these applications because, in addition to osteoprogenitor cells, they also enable endothelial cells to migrate into the matrix and develop the vascular beds necessary to nourish the newly formed tissue (Logeart-Avramoglou *et al.*, 2005). Particle size, shape and surface roughness affect cellular adhesion, proliferation and phenotype. Specifically, cells are sensitive and responsive to the chemistry, topography and surface energy of the material substrates with which they interact. In this respect, the type, amount and conformation of specific proteins that adsorb onto material surfaces, subsequently modulate cell functions (Boyan *et al.*, 1996). Calcium based ceramics undergo dissolution and precipitation at their surfaces. These events lead to the formation of a carbonate-containing hydroxyapatite layer, which promotes the attachment of bone forming cells (i.e., osteoconductivity) (Ohgushi *et al.*, 1999). Tricalcium phosphate (TCP) and hydroxyapatite (HA) are therefore most commonly used as a scaffold in bone tissue engineering. In addition, these two materials are commercially available from various producers (DeLong *et al.*, 2007) and well accepted in clinical practice as synthetic substitutes (or bone fillers).

In order to evaluate where is a niche for autologous cell therapy in medical practice, an overview of other established treatments is necessary.

2.1 Estabished treatments of bone defects

Orthopaedic trauma surgery requires the regular use of bone grafts to help provide timely healing of muscoskeletal injuries. The "perfect" bone graft has properties categorized as: osteoconductive, osteoinductive and osteogenic (De Long *et al.*, 2007). Osteoconduction is the property of a matrix that supports the attachment of bone-forming cells for subsequent bone formation. Osteoinduction is a process that supports the mitogenesis of undifferentiated cells, leading to the formation of osteoprogenitor cells that form new bone. The terms "osteogenic" and „osteogenesis" may be reserved for the ability to generate or the generation of bone by bone-forming cells.

2.1.1 Bone grafts and substitutes

Today, autologous bone grafting is the gold standard for osteogenic replacement in osseous defects (DeLong *et al.*, 2007). Autologous bone grafts reliably fill substance deficits and induce bone tissue formation at the defect site following transplantation. These grafts exhibit some initial stability, depending on donor site, size, shape and quality (Kneser *et al.*,

2006). However, the clinical use of autologous osseous transplants is limited by a considerable donor site morbidity, which increases with the amount of harvested bone. Bleeding, hematoma, infection and chronic pain are common complications of bone graft harvest (Ebraheim *et al.*, 2001). In addition, when the bone defect is large, there may not be enough autologous bone tissue to harvest. Processed allogeneic or xenogenic bone grafts are also commonly used for repair of osseous defects when autologous transplantation is not appropriate (Gazdag *et al.*, 1995). Although the initial properties of allogeneic or xenogenic grafts resemble those of autologous bone, the lack of osteogenicity is a limitation even when osteoinductive factors are preserved during processing. For specific indications, vascularized bone grafts from various locations, including fibula, scapula, iliac crest and others, are taken and transplanted into given bone defects (Ozaki et. al., 1997).

2.1.2 Synthetic bone substitutes

Degradable and non-degradable implant materials can be divided into synthetically produced metals and metallic alloys, ceramics, polymers and composites or modified natural materials (Mayer and Wiesmann, 2006). Whereas non-resorbable materials such as steel or titanium alloys are commonly used for prosthetic devices, resorbable substitute materials are currently being investigated for their use in bone and cartilage replacement therapies (Mayer and Wiesmann, 2006). Acrylate-based bone cements provide high mechanical stability after polymerization (Lewis, 1997). They are widely used for fixation of total joint prosthesis, vertebroplasty and for craniofacial bone defects. However, despite sophisticated modes of application, they do not possess osteogenic or osteoinductive properties and are slowly resorbed, if at all. Within the last two decades, many other biogenic and synthetic materials have been evaluated for their use as bone substitutes. Calcium phosphate- and apatite-based bone cements (porous composites and the most widespread ceramics used for bone reconstruction), as well as other types of biomaterials have been clinically applied for the treatment of fractures and bone defects (Jupiter *et al.*, 1997). Depending on their chemical composition and porosity, they are osteoconductive, biodegradable and are integrated into given bone defects (Kneser *et al.*, 2006).

2.2 Advanced approaches to treating bone defects

2.2.1 Osteoinductive substances

Although osteoinductive substances are clinically applied for the reconstruction of bone defects or for acceleration of fracture healing, only small numbers of patients have been treated and application modes and indications are not yet completely standardized. Platelet rich plasma contains, in addition to platelet derived growth factor (PDGF), a variety of different growth factors, depending on the processing and application modes andit enhances bone formation in experimental and clinical settings (Thornwarth *et al.*, 2006). Demineralized bone matrix (DBM) is prepared from allogeneic or xenogenic bone and is commercially available for clinical application in various formulations (Maddox *et al.*, 2000). Its osteoinductive potential is highly variable and depends not only on the donor but also on the processing protocols. DBM is commonly used in combination with other types of biomaterials. Bone morphogenetic proteins (BMPs) have been identified as the most relevant osteoinductive factor in demineralized bone matrix (Reddi *et al.*, 1998).

2.2.2 Allogenic bone tissue engineering products used in clinical practice and trials

The first registered tissue engineered bone product is called Osteocel, launched in the USA in July 2005 by Osiris Therapeutics Inc. It was the first product containing viable allogenic adult stem cells to be offered for the repair, replacement or reconstruction of bone defects. Osteocel promotes bone regeneration and is used to treat spinal defects or hard-to-heal fractures, in which the bone is shattered or pieces are missing. The producer declares this product to be the first bone matrix product to provide all three bone growth properties: osteoconduction, osteoinduction, and osteogenesis.

Osteocel is made from mesenchymal stem cells, which are mixed with spongy bone material obtained from human donors or cadavers. Because the cells are not manipulated (only harvested, processed and stored for later use, much like organs used for transplant), Osteocel is classified by the Food and Drug Administration (FDA) as a tissue transplant, not as a drug or a medical device. This product did not therefore have to go through the multiyear testing and approval process, which would most likely be be required for other stem cell products being developed.. Osteocel is an allogeneic tissue engineered product, exhibiting low immunogenicity and no activation of lymphocytes T in mixed leukocyte reaction testing *in vitro*. Osteocel grafts have been used since 2005 in over 30,000 procedures, with no reported adverse events.

Trinity™ by Blackstone Medical Inc. is another allograft substance that has recently begun to be used. Trinity BMSC are pre-immunodepleted and therefore do not stimulate local T-cell proliferation but instead are activated to act as osteoblasts and to stimulate bone formation. This local response can accelerate healing, earlier weight-bearing, healing and filling of bone voids in patients that have had excision of bony masses. In previous animal models, the use of BMSC has been shown to increase bone healing in critical sized defects. Trinity is currently approved by the FDA for use in trauma and bone defects within the spine, and has not shown any significant adverse effects compared with standard bone substitute products.

2.2.3 Autologous cell treatment approaches to bone defects

A widely accepted approach is the use of autologous cells for bone regeneration, which are frequently prepared as in-hospital procedures or produced only for the local market.

According to Chatterjea *et al.* (2010), 7 human clinical studies have so far been conducted based on the use of BMSC.

In 2001, BioTissue Technologies AG launched a product called BioSeed®-Oral Bone, using periosteum samples as a source of cells with osteogenic potential. BioSeed®-Oral Bone is a 3D jawbone graft used to reconstruct the jaw bone, for example in sinus lift operations or lower jaw augmentation.

Aastrom Biosciences in the USA has produced bone regeneration products for the treatment of osteonecrosis of the femoral head (called the ON-CORE trial) and a product for the treatment of severe non-union fractures (i.e., atrophic non-unions), both of which are in Clinical Phase III of development. Bone Repair Cells (BRCs) were derived from a small sample of the patient's bone marrow that is processed using Aastrom's Tissue Repair Cell (TRC) Technology to generate larger numbers of stem and early progenitor cells with

enhanced therapeutic potential. In the study, patients underwent standard open reduction and internal fixation surgery, in which BRCs were applied directly to the fracture site, together with an allograft bone matrix, to promote local bone regeneration. After the treatment with BRCs, patients with non-union tibia, humerus or femur fractures that had previously failed to heal after one or more "standard" medical procedures showed an overall healing rate of 91% after one year. The positive results from this study, together with early clinical data reported from osteonecrosis patients, further support the broad application of the proprietary TRC Technology in the field of orthopedics.

There have been several clinical reports about the treatment of critical-sized long bone defects with tissue engineering products using BMSC and scaffolds (Quarto *et al.*, 2001, Orozco *et al.*, 2005).

Some ongoing clinical trials are testing the treatment of non-union fractures and bone cysts by autologous mesenchymal stem cell percutaneous grafting as a minimally invasive implantation procedure (ClinicalTrials.gov Identifier: NCT01429012; NCT01206179; NCT01207193; NCT00916981; NCT00916981).

3. Testing different approaches for the production of autologous tissue engineered bone constructs

Aspects that need to be considered in planning a cell therapy/tissue engineering approach are:

- biological: cells to express adequate cell phenotype to produce bone tissue
- tissue engineering: scaffold that allows cell survival, is biodegradable, non-immunogenic, possesses appropriate biomechanical properties and is easy to handle
- surgical: adaption to the size and shape of the injury, a good clinical outcome could be expected upon the appropriate selection of clinical indications

Since 2005, we have been working on several bone tissue engineering projects employing various osteogenic cells:

- Engineering bone grafts using AO and rotating bioreactor – *in vitro study*
- Engineering bone grafts using BMSC- *in vitro study*
- Engineering bone grafts using ASC and perfusion culture - *in vitro study*
- Vascularization of tissue engineered bone grafts - *in vitro study*
- BMSC based bone grafts for the repair of long bone defects - *clinical project*
- Treatment of paradontal diseases with AO - *clinical project*

3.1 In vitro investigation of osteogenic potential of different cell sources

Various cell source, namely AO, BMSC, and ASCwere investigated in relation to different targeted clinical indications. The basic proof of osteogenic activity is mineralization of the extracellular matrix, which was found in all three investigated cell types. Additionally, specific gene expression and alkaline phosphatase activity was analyzed.

While AO were investigated to treat small volume defects in periodontal intrabony defects, both BMSC and ASC can be obtained in sufficient number from bone marrow aspirate or liposuction and proliferated enough to treat high volume bone defects (up to 50 cm^3) and

were studied in relation to the treatment of more extensive, e.g., long bone defects (pseudoarthrosis).

ASC and BMSC were tested for the expression of mesenchymal stem cell markers and for their capacity for mineralization after osteogenic differentiation. All tested cells expressed markers of mesenchymal stem cells DG73, CD90 and CD105 and were negative for CD34, which is a marker of hematopoietic cells (Table 1). All tested cell types were also positive for mineralization, which occurred in cultures of alveolar osteoblasts after 1-2 weeks of cultivation in osteogenic medium and in cultures of both mesenchymal stem cell types after 2-3 weeks of cultivation in osteogenic medium (Fig. 1). The intensity of matrix mineralization, however, significantly varied among cell cultures from different donors.

Marker / Sample	CD 105	CD 90	CD 73	CD34
BMSC 30	+	+++	+++	---
BMSC 31	+	+++	+++	---
BMSC 35	+	+++	+++	---
ASC 01 (P3)	+	+++	+++	---
ASC 02 (P3)	++	+++	+++	---
ASC 03 (P3)	+	+++	+++	---
ASC 04 (P6)	+++	+++	+++	---

Table 1. Expression of mesenchymal stem cell markers by BMSC and ASC

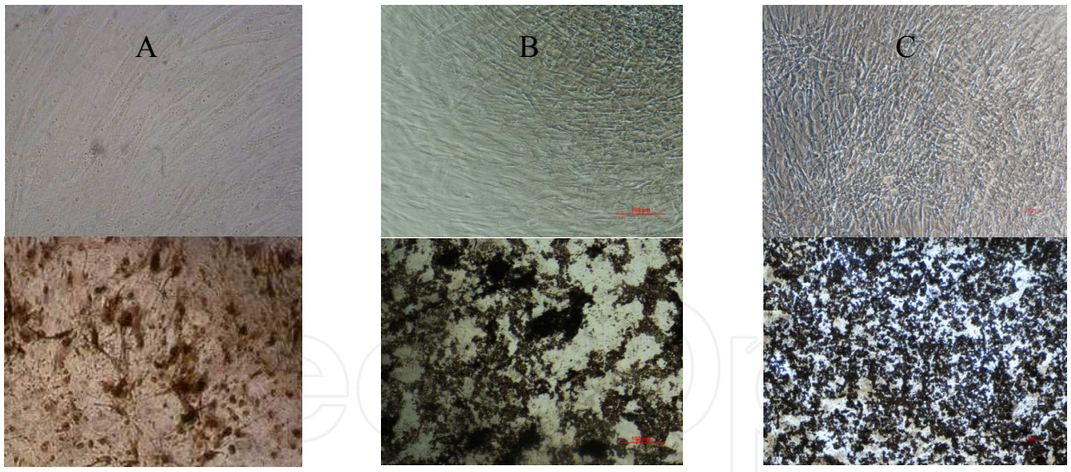

Fig. 1. Mineralization of the matrix is occurring in cultures of AO (A), BMSC (B) and ASC (C) after induction of osteogenic differentiation (upper line: culture in normal medium, bottom line: cultures in osteogenic medium). Von Kossa staining. (photos were taken at a magnification of 100x).

3.1.1 Engineering bone grafts using Alveolar Osteoblasts (AO) and a rotating bioreactor

AO can be isolated from alveolar bone tissue that is normally discarded prior to treatment of periodontal diseases. The use of alveolar bone tissue as a cell source for periodontal

indications therefore represents no additional harm to the patient and is thus considered to be the optimal cell source for this application.

The aim of this study was to engineer bone grafts using AO for treating bone degeneration in periodontal diseases.

After harvesting a piece (approx. 40 mm^3) of maxillar or mandibular alveolar bone, primary explant culture and subsequently cell cultures of the first passage were established (Fig. 2).

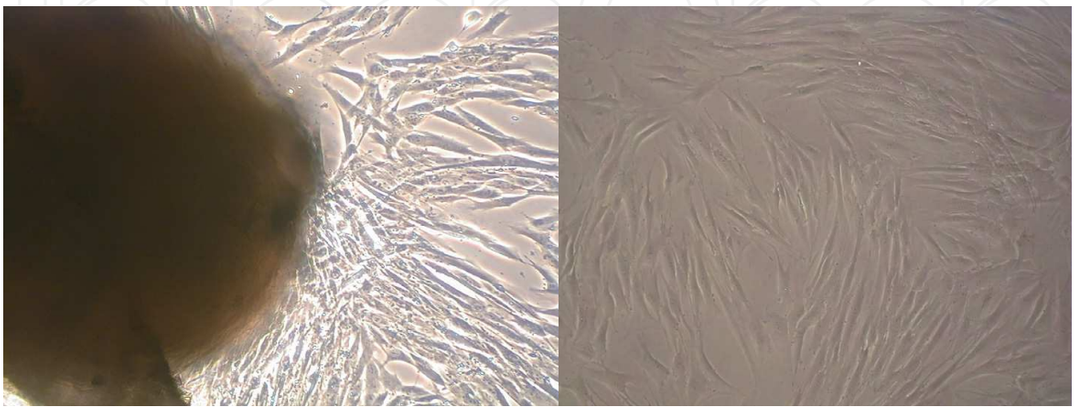

Fig. 2. Primary explant culture (left) and the first passage (right) of AO cells for the treatment of periodontitis (photos were taken at magnification 100x).

Expanded AO with proven osteogenic potential were loaded onto macroporous hydroxyapatite granules together with fibrin glue, which enabled the formation of solid grafts (Fig. 3), and cultured in medium supplemented with osteogenic differentiation factors for up to three weeks in a rotating bioreactor. Light and scanning electron microscopic examinations of the cell-seeded constructs showed a uniform cell distribution, as well as cell attachment and growth into the interior region of the hydroxyapatite granules (Fig. 4). Cells in tissue constructs exhibited growth patterns of enhanced proliferation during the first two weeks of cultivation, followed by a decrease in cell numbers.

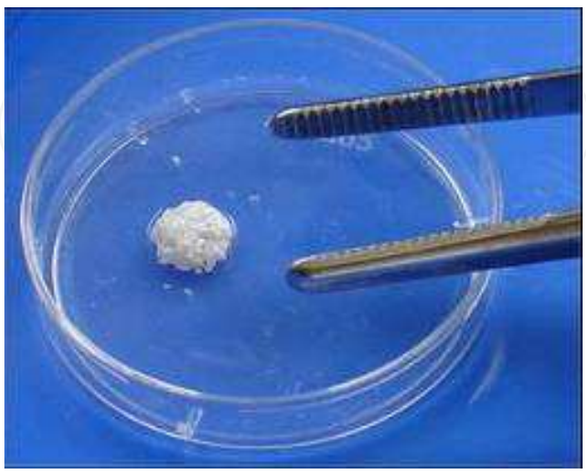

Fig. 3. Bone tissue engineered graft for the treatment of periodontal intrabony defects, macroscopic view of the graft.

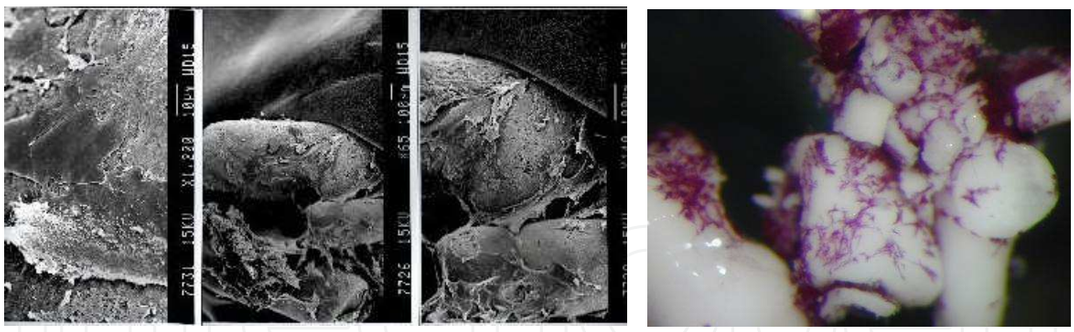

Fig. 4. AO growing over the hydroxyapatite granules of the scaffold one week after graft formation A: scanning electron microscopy, B: stereomicroscope (cells stained with MTT)

The osteogenic potential of the cells was tested by observation of the mineralisation capacity and analysis of gene expression of three important marker genes for osteogenesis: alkaline phoshatase, osteopontin and osteocalcin. Alkaline phosphatase activity was higher at three weeks in all cultures in osteogenic medium than in the control medium. Gene expression levels exhibited patterns of osteogenic differentiation (Maličev *et al.,* 2008).

We showed that bone-like constructs with viable cells exhibiting differentiated osteogenic phenotype can be prepared by cultivation of AO on hydroxyapatite granules.

3.1.2 Engineering bone grafts using Bone Marrow derived Stem Cells (BMSC)

Bone Marrow derived Stem Cells (BMSC) – also termed mesenchymal stem cells (MSC) or multipotent adult progenitor cells (MAPC) – are progenitors of skeletal tissue components such as bone, cartilage, muscle, the hematopoiesis-supporting stroma and adipocytes (Pittenger *et al.*, 1999; Flanagan *et al.*, 2001). The development of methods for isolation, expansion and controlled differentiation of BMSC offers possibilities of using these cells as an integral component of various clinical applications of tissue engineering, especially in reparative osteogenesis.

The aim of this study was to engineer bone grafts using BMSC for treating long bone defects in patients with pseudoartrosis.

After harvesting bone marrow from the iliac crest (approx. 30 ml), mononuclear cells were separated by gradient centrifugation and seeded in primary culture. Non-adherent cells were washed out after 24 hours and adherent cells were expanded and passaged to obtain a sufficient number of cells.

Osteogenic differentiation was carried out in confluent monolayer cultures of the second passage, which was confirmed by positive von Kossa staining (calcium deposits) and staining for the enhanced presence of alkaline phosphatase (Fig. 5). In addition, higher gene expression levels of bone sialoprotein II, osteopontin and BMP2 were determined in BMSC after osteogenic differentiation compared to control BMSC.

Porous TCP granules were used as a scaffold. The cells were seeded directly onto the granules to achieve an approximate total of 1×10^6 cells per 1 mL of the tissue engineering bone construct. The granules were "glued" by inducing fibrin clot formation with the addition of thrombin (Fig. 6). Cell viability in the tissue bone construct was confirmed by

MTT staining. Light microscopy examination of the cell-seeded constructs showed a uniform distribution of viable cells (Fig. 7).

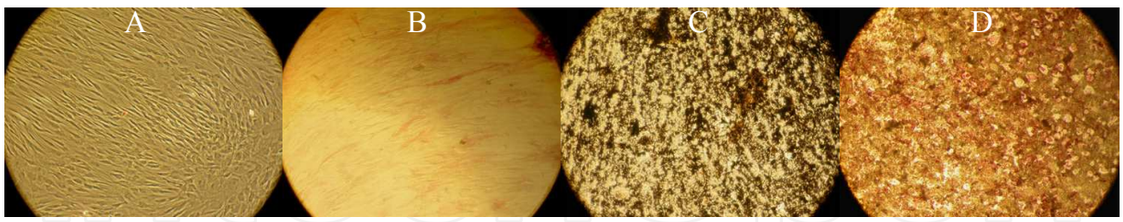

Fig. 5. Second passage of BMSC after 18 days in basal growth medium (A,B) and in osteogenic medium (C,D), respectively. The cells were subsequently stained for calcium deposits according to von Kossa (A,C) and for alkaline phosphatase (B,D) (photos were taken at magnification 100x).

Fig. 6. Preparation of bone implant composed by BMSC after osteogenic differentiation, TCP granules and fibrin glue.

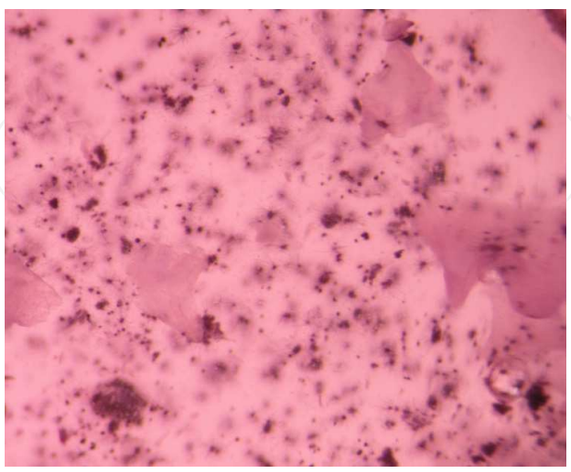

Fig. 7. BMSC after osteogenic differentiation and seeding onto the granules of TCP and staining with MTT, showing an equal distribution of cells in the graft (stereomicroscope, photos were taken at magnification 20x).

3.1.3 Engineering bone grafts using Adipose-derived Stem Cells (ASC) in a perfusion bioreactor

ASC are an attractive cell source for autologous bone tissue engineering, due to their easy accessibility and abundance, as well as their potential for osteogenic differentiation (Zuk *et al.*, 2001). In combination with scaffolds with mechanical properties similar to native bone, they could enable engineering of bone grafts for treating load-bearing sites.

The aim of this study was to engineer bone grafts using ASC on decellularized bone scaffolds and to evaluate the effects of long term perfusion culture conditions (enabling efficient cell nutrition and gas exchange) on the quality (cell distribution and bone matrix formation) of bone grafts. Perfusion culture has already been proved to be beneficial for BMSC based grafts in terms of cell distribution and bone matrix deposition (Gomes *et al.*, 2003; Grayson *et al.*, 2008).

Human ASC were isolated from lipoaspirates of three different donors, characterized and expanded up to the 3rd passage. The osteogenic potential of ASC was tested using von Kossa and Alizarin Red staining. For the perfusion study, cells were seeded on decellularized bovine trabecular bone scaffolds (4 mm ∅ × 4 mm) and subsequently cultured in two different medias (control and osteogenic), in static culture and perfusion bioreactors (Fig. 9). Four experimental groups were formed: (*i*) control-static, (*ii*) control-perfused, (*iii*) osteo-static and (*iv*) osteo-perfused. After 5 weeks, constructs were evaluated for cell viability (live/dead assay), DNA content (PicoGreen), cell distribution (4'-6-diamidino-2-phenylindole - DAPI), collagen (Trichrome), osteopontin and sialoprotein (immunohistochemistry).

ASC from three different donors showed that osteogenic culture conditions resulted in strong mineral deposition, as confirmed by von Kossa and Alizarin Red staining. Additionally, these data show a significant donor-to-donor variability in the osteogenesis of ASC (Fig 8).

During cultivation of ASC grafts, the DNA content increased in all experimental groups and was generally higher under osteogenic than under control conditions. Histological analysis demonstrated that grafts cultured in osteogenic medium contained more total collagen, bone sialoprotein and osteopontin than matching controls. Additionally, under static culture conditions, cell growth and matrix deposition were located mostly at the construct periphery, while perfused constructs exhibited a more even cell and matrix distribution throughout the scaffold volume (Figs. 10 and 11).

In summary, a combination of ASC as cell source, decellularized bone as scaffold and perfused culture conditions in combination with osteo-inductive supplements, provides a promising approach to obtaining high quality tissue engineered bone grafts. Furthermore, cultivation of ASC in a perfusion bioreactor improves cell and bone matrix distribution within the graft and therefore assures a superior cultivation environment to static culture, especially for larger grafts and for longer periods of time. However, for the successful application of ASC based bone grafts in clinical settings, the donor-to-donor variability in the osteogenic potential of ASC needs to be considered. (Fröhlich *et al.*, 2010)

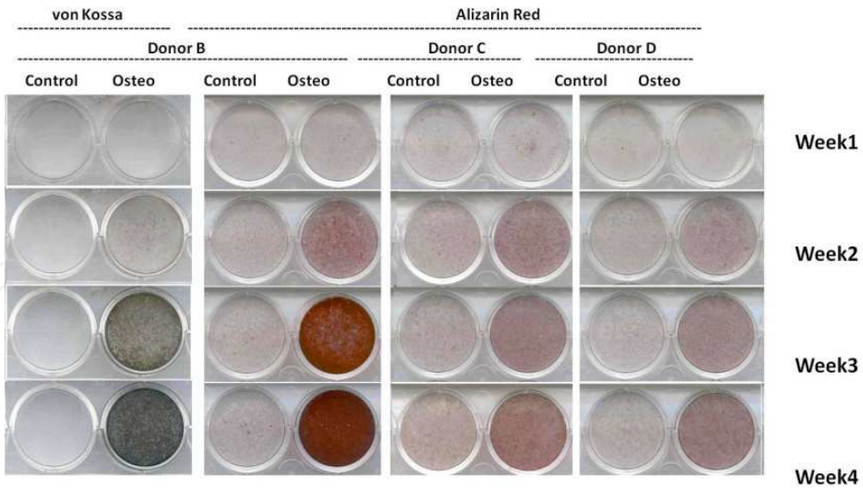

Fig. 8. Osteogenic potential of ASC. ASC of three donors (B, C, D) were cultured under either control or osteogenic medium for various time lengths and were stained with Alizarin Red (red) and von Kossa (black). (Fröhlich *et al.*, 2010)

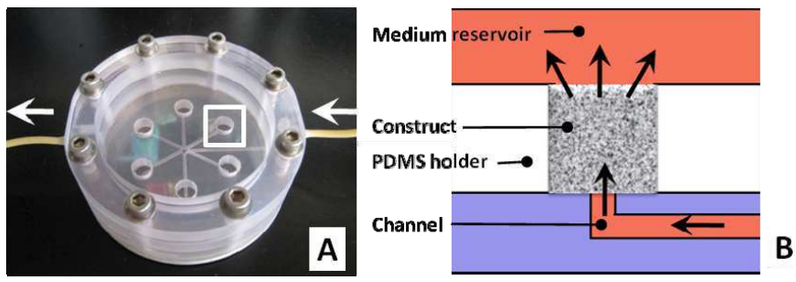

Fig. 9. Perfusion bioreactor used in the study. The region indicated by a white rectangle in A is shown schematically in panel B. Medium flows throughout the scaffold, as indicated by arrows (B). (Fröhlich *et al.*, 2010)

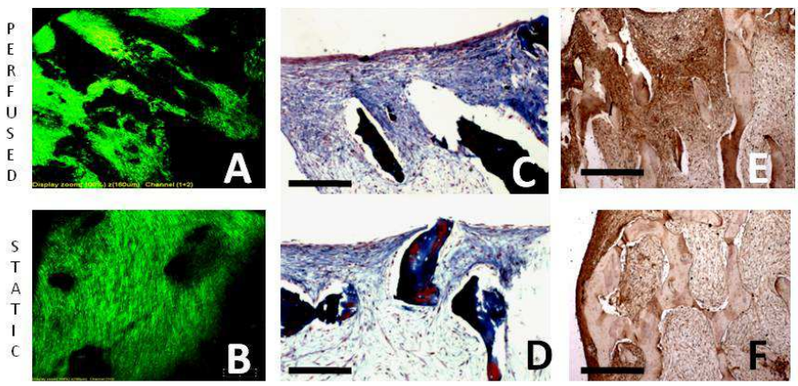

Fig. 10. Long term cultivation of ASC grafts in static and perfused culture. Live/dead staining of the central part of the cultured grafts under perfused (A) and static (B) conditions. Collagen (C, D) (blue) and osteopontin (E, F) (brown) deposition within the scaffold is more abundant and more uniformly distributed under perfused conditions (C, E) than under static culture (D, F). The scale bar is 0.5 mm. (Fröhlich *et al.*, 2010)

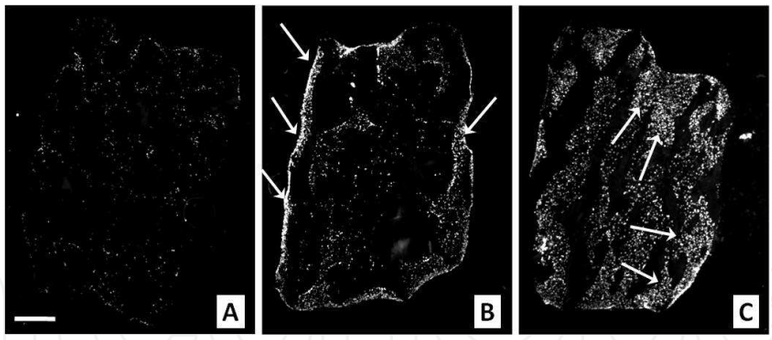

Fig. 11. Cell distribution within the ASC bone grafts. Constructs were stained with DAPI to visualize cell distribution (cell nuclei shown in white). Seeding resulted in an even initial distribution of cells throughout the scaffold (A). After 5 weeks of static culture, cells were found mostly in the outer regions of the constructs (indicated by arrows) (B). After 5 weeks of culture with medium perfusion, cells were more evenly distributed throughout the construct volume (indicated by arrows) (C). The scale bar is 0.5mm. (Fröhlich *et al.*, 2010)

3.2 Vascularization of tissue engineered bone grafts

Vascularization is of critical importance for the integration and survival of larger engineered bone grafts on implantation, since it ensures efficient gas and nutrition exchange with all cells within the tissue.

There are several approaches being utilized in order to vascularize bone grafts, and generally one or a combination of three major principles can be followed (Fig. 12). *In vivo* pre-vascularization employs the implantation of the bone grafts into environments rich in vascular supply (subcutaneous, intramuscular or intraperitoneal sites), where the constructs can be invaded with new vascular networks at their surfaces. However, transplantation to the site of interest is impossible without damaging the initial vascular network. Vascularization of an implanted graft can also be accelerated by the utilization of angiogenic factors. Growth factors, such as VEGF, PDGF and FGF, play a crucial role in angiogenesis (Jain *et al*., 2003). Incorporation of these factors into scaffolds and control of their local release rate and delivery regime is one possibility for accelerating vascular in-growth *in vivo*. Another way of achieving vascularization of tissue engineered bone grafts is co-culturing endothelial and osteogenic cells into bone constructs engineered *in vitro* – the so called *in vitro* prevascularization approach. Endothelial cells have the potential to form new vessels within the scaffolds, with the potential to anastomose with the host vasculature when implanted *in vivo*. Moreover, endothelial cells not only contribute to forming the vasculature to deliver nutrients to the bone but are also important in terms of interaction with and differentiation of osteoprogenitor cells (Rouwkema et al., 2006; Unger *et al*., 2007). Adult endothelial cells can be used as a source of endothelial cells, but recently, adult mesenchymal stem cells have also been shown to have the potential to differentiate toward the endothelial lineage (Miranville et al., 2004; Valarmathi et al., 2008). (Reviewed in Fröhlich *et al.*, 2008).

In addition to endothelial cells, smooth muscle cells or pericytes are also necessary for forming a functional vasculature. We exploited the vasculogenic potential of ASC and showed that ASC spontaneously, as well as in induced cultures, formed up to 1 mm long

endothelial structures. In the same manner, ASC had the potential for smooth muscle phenotype (Fig. 13). (Fröhlich *et al.*, 2009) Since they have all the necessary types of cells - osteogenic (Fig. 8, Fig. 9) and vascular (Fig. 13), ASC seem to be an ideal source of cells for engineering autologous vascularized bone grafts. However, optimal culture conditions for the co-existence of various cell types still need to be determined.

BMSC have also been tested for their smooth muscle and endothelial phenotype. BMSC expressed α smooth- muscle actin characteristic of smooth muscle cells (Fig. 14), but did not form endothelial structures, as seen with ASC (data not shown).

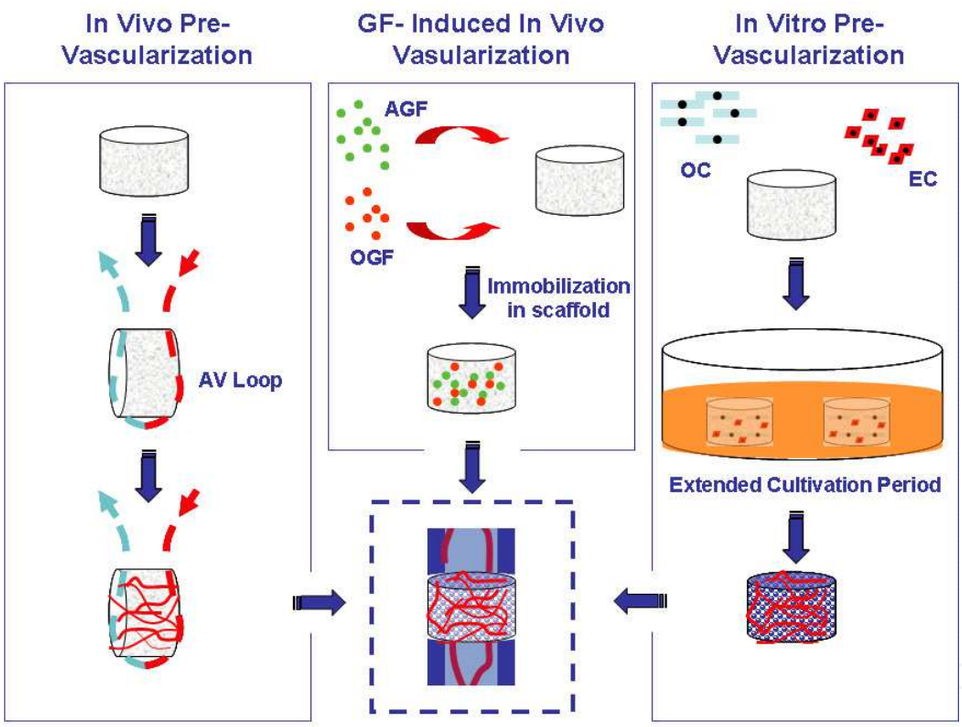

Fig. 12. Approaches to vascularizing engineered bone scaffolds. *Left:* The arterio-venous (AV) loop as an example of an *in vivo* approach for pre-vascularizing scaffolds. **Center:** One cell-free approach is to immobilize angiogenic growth factors (AGF) and osteogenic growth factors (OGF) in scaffolds and directly implant into the site of interest. In this method, the growth-factors induce migration of angiogenic and osteo-progenitor cells and provide them with the stimuli for neo-vessel formation and osteogenic differentiation. **Right:** The cell-based, tissue-engineering approach utilizes osteogenic cells (OC) and endothelial cells (EC) in a three-dimensional co-culture. (Fröhlich *et al.*, 2008)

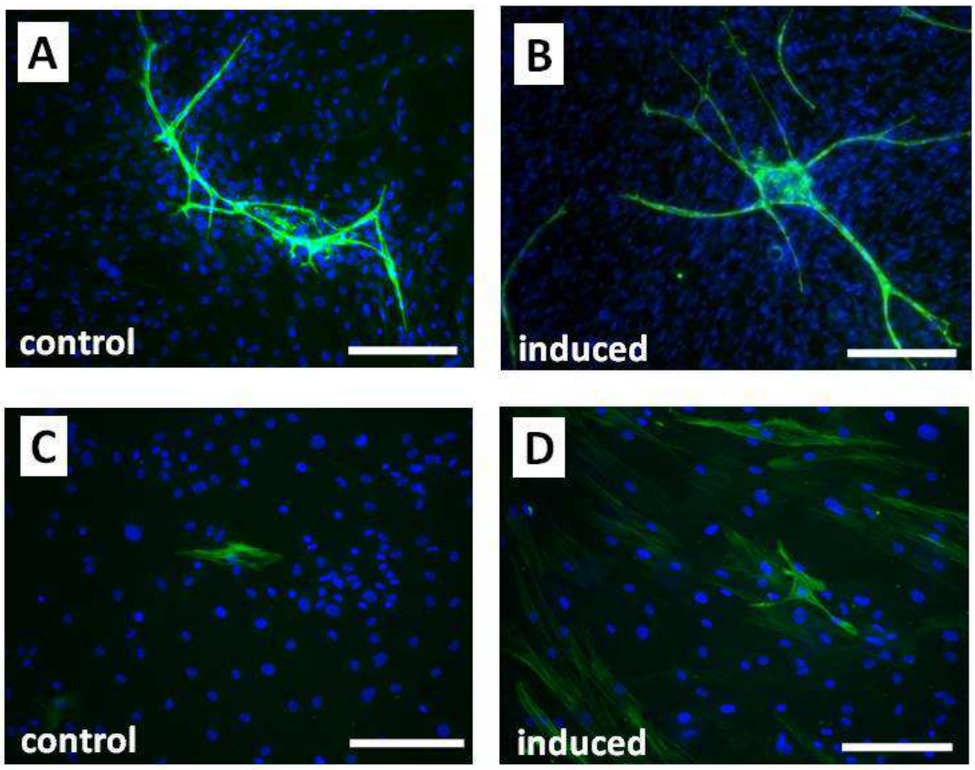

Fig. 13. ASC as a cell source for vascularization of bone constructs. ASC formed up to 1 mm long CD31 positive endothelial structures (green) with *the* close proximity of surrounding cells (blue stained nuclei) when cultured in stromal (control) medium (A). ASC also formed endothelial structures in endothelial medium *but* the structures were less numerous and without the specific pattern of surrounding cells (B). After induction with smooth muscle medium, *the* number of α smooth-muscle actin positive cells (green) increased (D) in comparison to *the* control medium (C). *The s*cale bar is 200 μm. (Fröhlich et al., 2009)

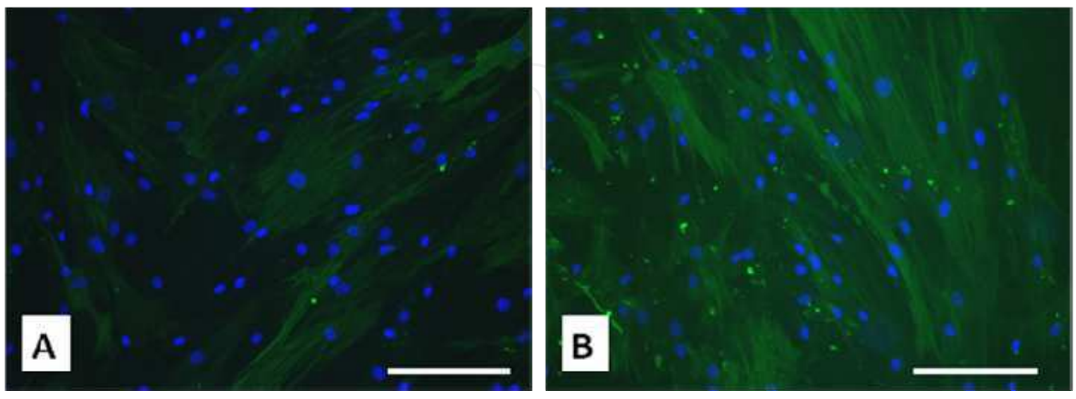

Fig. 14. BMSC of 3rd passage were induced to differentiate into the smooth muscle phenotype. Positive staining for α smooth-muscle actin was evident in the control medium (A) and was further enhanced by exposing the cells to induction medium (B). *The scale bar is 200 μm.*

3.3 Clinical projects for bone regeneration

The first concept (Section 3.1.1.) - employing osteoblasts of cancellous alveolar bone loaded onto the HA granules - has been developed to treat periodontal diseases (BoneArt™-A). The second approach (Section 3.1.2.) - employing bone marrow derived mesenchymal stem cells (BMSCs) differentiated into osteoblasts and loaded onto the TCP granules - has been developed to treat long bone defects (BoneArt™-S). In both cases, the principle is similar (Fig. 15): cells are isolated and proliferated from autologous tissue harvested from patients. Using a criopreservation step, we can adapt to the time of predicted implantation. When cells are proliferated to the desired number, they are seeded on scaffold material. Induction of differentiation can be added to the protocol before or after bone graft preparation. The bone graft can either be implanted immediately or submitted to conditions that stimulate osteogenesis prior to implantation. Grafts need to be tested according to the quality control (QC) protocol, ensuring the safety and efficiency of the product.

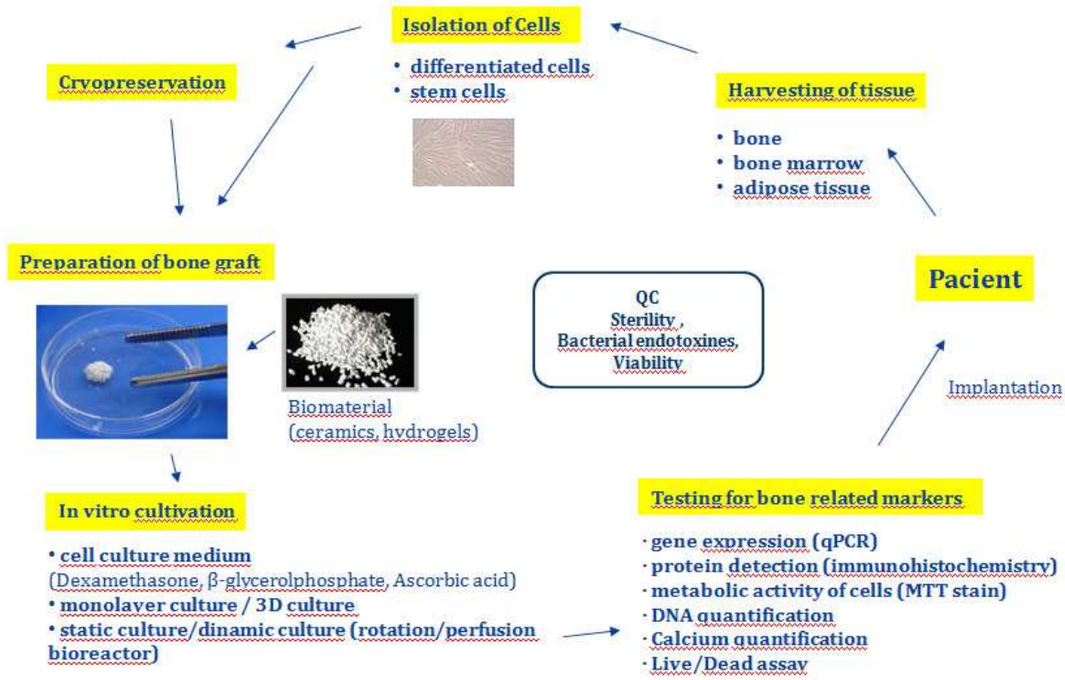

Fig. 15. Principle of bone tissue engineering for clinical application.

3.3.1 Autologous Alveolar Osteoblasts (AO) for the treatment of paradontosis

Periodontal diseases (periodontitis) is a chronic, infectious, inflammatory disease that affects the dental attachment apparatus - i.e. the tissues that support and anchor the teeth to the jaw; these include the cementum, periodontal ligament ans alveolar bone. If left untreated, periodontal disease may result in complete destruction of the alveolar bone as well as the othe supporting tissues. (Lin *et al.*, 2008).

The possibility of enhancing bone regeneration by implanting alveolar osteoblasts (AO) in combination with an appropriate scaffold is of clinical interest, particularly in reconstructive maxillofacial surgery and periodontology (Lin et al., 2008). In the research project, the

concept was tested that artificial matrices, seeded with cells of osteogenic potential, may be implanted into sites where osseous damage has occurred, which could lead to significant osseous regeneration.

Firstly, the growth and differentiation of alveolar bone cells in tissue-engineered constructs and in monolayer cultures, as a basis for developing procedures for routine preparation of bone-like tissue constructs, were compared (Maličev *et al*., 2008).

Autologous constructs as described above (Section 3.1.1) were prepared to treat six patients with aggressive periodontitis by an implantation of a cell-based alkaline phosphatase approach. The operative implantation procedures were carried out without any complications and no side effects were detected that could be assigned to the tissue engineered construct. The newly forming bone is clearly seen in X-rays 3 months after implantation (Fig. 16). Clinical evaluation at 6 months and 12 months after implantation showed a significantly higher gain of clinical attachment in cases in which cellularized grafts were implanted in comparison with the control group (implantation of the scaffold alone) (Fig. 17).

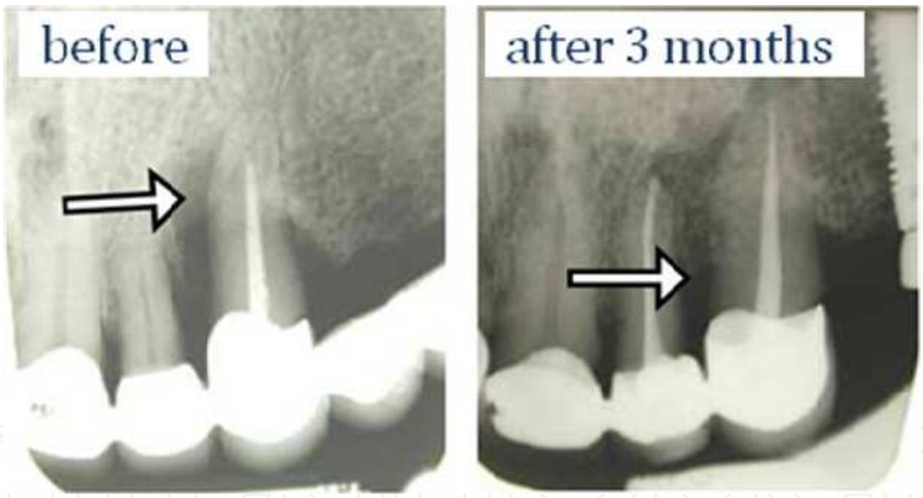

Fig. 16. X-ray of implanted site before and 3 months after treatment. Arrows indicate the limit of bone tissue.

In the first observation period after implantation, there was a significantly higher gain of clinical attachment in sites at which cells were added, compared to sites at which only material was implanted, while no difference is observed in the second period. Overall, in cases in which cells were implanted together with biomaterial, the bone regeneration process was faster and more efficient.

This clinical project confirmed the positive effect of autologous cell therapy for bone regeneration.

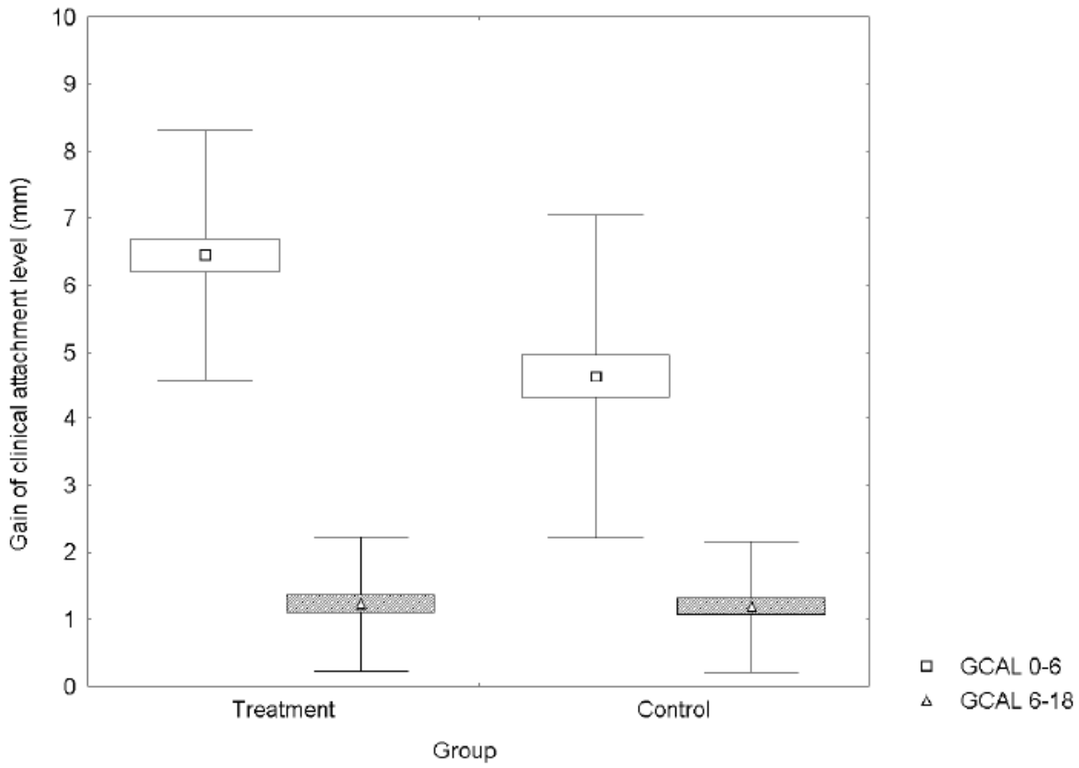

Fig. 17. Gain of clinical attachment measured up to 6 months after implantation (GCAL 0-6) and gain of clinical attachment in the period from first measurement to second measurement up to 18 months after implantation.

3.3.2 Autologous Bone Mesenchymal Stem Cells (BMSC) for the treatment of large long bone defects

A project to evaluate the concept of bone tissue engineering for the treatment of severe long bone defects was carried out using autologous BMSC differentiated into osteoblasts as a cell source and a TCP scaffold in combination with fibrin glue (Figs. 5-7). The bone marrow was harvested from the patient's posterior iliac crest. BMSC were isolated and expanded to the desired number according to Pittinger *et al.,* (1999) with some modifications as described in 3.1.2 (Krečič Stres *et al.*, 2007). Expanded cells with proven osteogenic potential were loaded onto macroporous TCP granules together with fibrin glue, which enabled the formation of solid grafts (Fig. 6). An outline of the procedure for the preparation of tissue engineered bone graft is shown in Figure 18.

The tissue engineered bone construct was surgically implanted to fill gaps in the long bone of patients, mainly for the treatment of pseudoarthrosis in the femur or tibia.

Six patients with a history of multiple failing surgical revisions were treated according to the described procedure (Fig. 19A). None of the patients had any side affects connected with the treatment procedure. Preliminary results were promising since they suggested ossification of the bone defects on X-ray (Fig. 19 B,C). Scintigraphy (^{99m}Tc DPD) also showed evident perfusion and osteoblast activity in the implanted site. At the intermediate observation (5-14

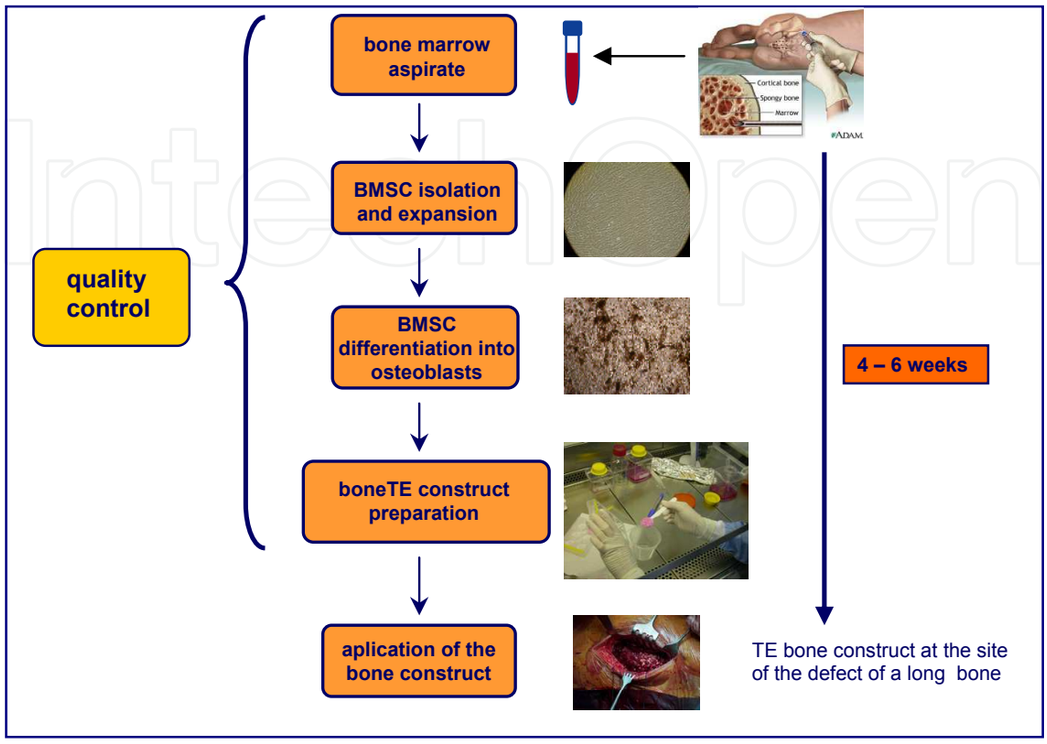

Fig. 18. An outline of the procedure for the preparation of tissue engineered bone graft BoneArt™ for long bone defect treatment from autologous BMSC as carried out in Educell Ltd.

months after implantation), bone bridging or callus formation was observed in 4 out of 6 patients and 3 patients were allowed full weight bearing of the treated leg.

However, the final evaluation of the clinical outcome did not show the expected results. Factors that probably contributed to the failure of these treatments were:

- the extensive volume of the missing tissue (up to 50 ml), which hindered perfusion of the graft as it was designed
- damaged/inadequate surrounding tissue (fibrotic tissue after burns…)
- septic events prior to cell implantation

Due to the small number of patients included in our study, as well as their clinical history, we cannot reach general conclusions about how useful a cell based treatment approach could be in the treatment of non-unions.

Several clinical reports do show successful results of implantation of tissue engineering bone tissues although, especially in large defects, in which a tissue engineering approach is expected to help after other treatments have failed, probably more advanced treatment concepts, considering also perfusion and vascularisation of the tissue, should be developed.

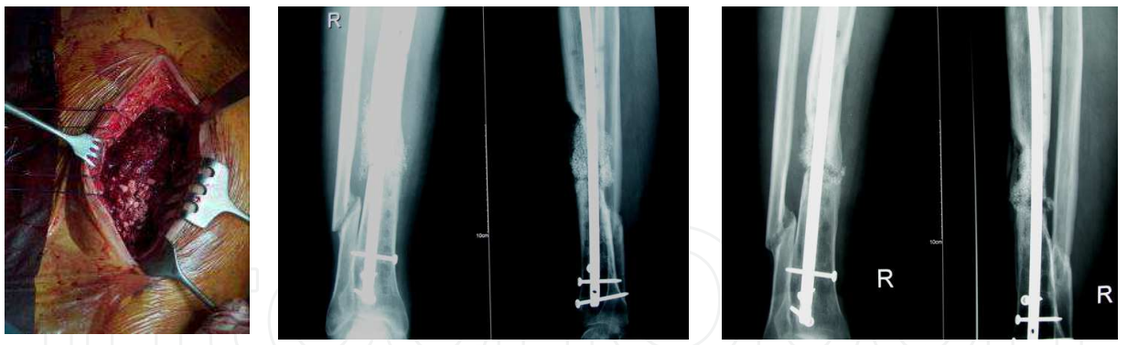

Fig. 19. Clinical application of tissue engineered bone graft. A: implantation of the graft, B: X-ray immediately after implantation, granulation of the TCP in a graft is visible, C: X-ray 6 months after implantation, the formation of new bone can be observed on the proximal part of the tibia, however complete bone filling was not achieved and a defect remained on the distal part of the non-union

4. Conclusion

Despite the high regeneration capacity of bone tissue, surgical procedures used in reparative osteogenesis do not consistantly result in structural and functional recovery. This state is associated with the disintegration or insufficiency of cambial cells in bone tissue and osteogenic deficiency. Cell based therapies are a new therapeutic approaches in regenerative medicine and using autologous cells is a promising strategy for bone regeneration.

We tested the three of the most studied and relevant sources of osteogenic cells: osteoblasts (from alveolar bone), bone marrow derived stem cells (BMSC) and adipose derived stem cells (ASC). We showed that all three cell sources posses adequate proliferation capacity for potential tissue engineering applications and their differentiation capacity was also proven by testing mineralisation of the extracellular matrix as well as gene expression, specific for osteogenic differentiation.

However, clinical application of a tissue engineering approach is not reflecting the enormous effort in research and preclinical development that has been invested so far - there is still a severe, unmet need for technologies that will facilitate bone tissue regeneration.

Our clinical projects indicate a positive effect of cell based therapies for the treatment of bone defects; in the case of alveolar bone tissue as well as in the case of long bone defects. However, there are limitations in the technology, especially in the treatment of large defects.

Extensive research on tissue vascularization might help cell and tissue engineering technologies become more prospective in bone regeneration. From this aspect, the vascular potential of mesenchymal stem cells seems to indicate a promising area for further bone tissue vascularization research.

Although basic research on osteogenic differentiation potentials of stem and other osteogenic cells is crucial for understanding the bone tissue engineering area, and promises great potential for its use in clinics, only experience from clinical applications will give

relevant information and final answers regarding the usefulness of cell and tissue engineered products for various clinical indications.

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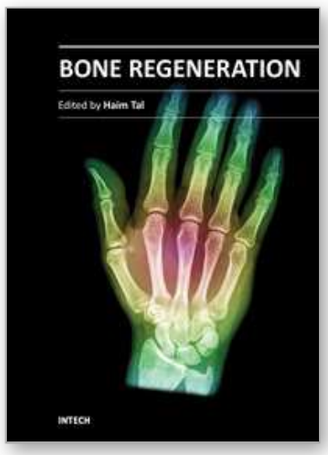

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Bone is a specialized connective tissue, most prominently characterized by its mineralized organic matrix that imparts the physical properties that allow bone tissue to resist load, to support functional organs, and to protect highly sensitive body parts. Bone loss and bone damage may occur as a result of genetic conditions, infectious diseases, tumours, and trauma. Bone healing and repair, involves integrative activity of native tissues and living cells, and lends itself to the incorporation of naturally derived or biocompatible synthetic scaffolds, aimed at replacing missing or damaged osseous tissues. There are several modalities of bone regeneration including tissue engineering, guided bone regeneration, distraction ontogenesis, and bone grafting. This book concentrates on such procedures that may well be counted among the recent outstanding breakthroughs in bone regenerative therapy.

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