

**Quantitative and qualitative assessment of the
regenerative potential of osteoblasts versus
bone marrow derived mesenchymal progenitor
cells in the reconstruction of critical sized
segmental tibial bone defects in a large animal
model**

Arne Berner, MD

Faculty of Science and Engineering, Queensland University of Technology

Thesis submitted for:

Doctor of Philosophy (PhD)

2013

Keywords

Tissue engineering, bone regeneration, segmental bone defect, tibia, scaffold, tricalcium-phosphate, mesenchymal progenitor cells, bone morphogenetic protein, preclinical animal model

Abstract

Bone defects caused by trauma, revision surgery, inflammation, tumour surgery and developmental deformity remain a major challenge for orthopaedic surgeons. There are several different treatment methods available, but no consensus guidelines exist and the treatment of such defects differs greatly. In recent years, bone grafts have advanced as the “gold standard” treatment to augment or accelerate bone regeneration. There are however, significant drawbacks associated with this approach. Therefore, several clinicians and researchers are investigating novel tissue engineering concepts to treat large bone defects.

The use of mesenchymal progenitor cells in cell-based strategies has emerged as a promising alternative in regenerative medicine. In the past, bone marrow mesenchymal progenitor cells (MPC), osteoblasts isolated from the axial skeleton (tOB) and osteoblasts isolated from orofacial bones (mOB) have been suggested for the enhancement of bone repair.

In this study, the osteogenic potential of different cell types and different cell applications in combination with a novel, medical grade composite scaffold were compared in a clinically relevant critical-sized tibial bone defect in a large animal model.

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List of Abbreviations

2D	Two dimensional
3D	Three dimensional
ABG	Autologous bone graft
ALP	Alkaline phosphatase
Ang 1	Angiopoietin 1
AO	Arbeitsgemeinschaft Osteosynthese
ARC	Australian Research Council
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BMSC	Bone marrow derived stem cells
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
BV	Bone volume
CAD	Computer aided design
CD	Cluster of differentiation
CFU	Colony forming unit
CO ₂	Carbon dioxide
CSD	Critical sized defect
DAPI	4',6-diamidino-2-phenylindole
DCP	Dynamic compression plate
DMEM	Dulbecco's modified eagle media
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylene-di-amine-tetra-acetic acid
EDX	Energy dispersive X-ray spectroscopy
EGF	Epidermal growth factor
EU	European union
f	frequency
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FDA	Fluorescein diacetate
FDA	Food and drug administration
FDM	Fused deposition modelling
FEM	Finite element modelling
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GAGs	Glycosaminoglycans
GDF	Growth differentiation factor
H ₂ O	water
HCl	Hydrogen chloride
HE	Haematoxylin-eosin
HGF	Hepatocyte growth factor
IGF-1	Insulin like growth factor 1
ISO	International organization for standardization
KGF	Keratinocyte growth factor

LC-DCP	Limited contact dynamic compression plate
LISS	Less invasive stabilization system
MIP 1	Macrophage inflammatory protein 1
MPC	Mesenchymal progenitor cell
mPCL	medical grade poly-caprolactone
MSC	Mesenchymal stem cell
MPC	Mesenchymal progenitor cells
MVF	Mineralized volume fraction
(μ)CT	(Micro) computed tomography
NaOH	Sodium hydroxide
OB	Osteoblast
OC	Osteocalcin
OP-1	Osteogenic protein 1
PBS	Phosphate buffered saline
PC-Fix	Point contact fixator
PCL	Poly-caprolactone
PDGF	Platelet derived growth factor
PDLA	Poly-D-lactic acid
PEG	Polyethylene glycol
PGA	Poly-glycolic acid
PLA	Poly-lactic acid
PLLA	Poly-L-lactic acid
PMMA	Poly-methyl-methacrylate
pMOI	Polar moment of inertia
PRP	Platelet rich plasma
rh	Recombinant human
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SD	Standard deviation
SDF 1	Stromal derived factor 1
SBF	Solution Body Fluid
TCP	Tricalcium-phosphate
TEC	Tissue engineered construct
TGF	Transforming growth factor
TRAP	Tartrate resistant acid phosphatase
UHN	Universal humeral nail
UTN	Universal tibial nail
VEGF	Vascular endothelial growth factor
XPS	X-ray photoelectron spectroscopy

Statement of Original Authorship

“The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.”

QUT Verified Signature

Wednesday, 04 February 2013

Acknowledgement

I would like to gratefully acknowledge Prof. Dietmar W. Hutmacher for his friendship and enthusiastic supervision of my work and the time in his Institute in Brisbane, Australia. I thank Prof. Michael Schütz for his support, critical discussions, and supervision of my work.

Furthermore, I would like to thank Dr. Maria Ann Woodruff, Dr. Siamak Saifzadeh, Dr. Johannes Reichert, Dr. Verena Reichert, Dr. Roland Steck, Dr. Ferry Melchels, Dr. Mostyn Young, Dr. Brendan Jones, Mr. Sascha Zaiss, Mrs. Alyssa Waite, Mr. Edward Ren and the team of the QUT Medical Engineering Research Facility for their friendship, advice, help with the experiments and help and assistance with the animal surgeries.

Thank you to the members of the IHBI Regenerative Medicine Group and my fellow research students for companionship, inspiration and advice.

Finally, I would like to thank my wife Annalena for her love and support and my two sons Leonard and Maximilian for their patience to share “Daddy” with the Institute.

At least I would like to thank my parents and siblings for support and encouragement over the years.

Chapter I - Introduction

1.1. Clinical background

In most cases, bone possesses a good healing capacity and the vast majority of bone defects, when stimulated by well-balanced biological and micro-environmental conditions, heal spontaneously. Refinements in surgical techniques, implant design and peri-operative management have significantly improved the treatment of complex fractures and other skeletal defects caused by high energy trauma, disease, developmental deformity, revision surgery and tumour resection^{1,2,3}. However, an unfavourable wound environment, sub-optimal surgical technique or biomechanical instability can lead to formation of large defects with limited intrinsic regenerative potential⁴. Such defects pose a major surgical, socio-economical and research challenge and can highly influence the patient's quality of life due to limb length discrepancy and prolonged, postoperative treatment courses^{5,6}. There are different therapeutic options for bone non-unions and bone defects in current clinical use, including both operative and non-operative treatments. So far, there is no consensus in the guidelines and the treatment of such defects differs, depending on the skills of the surgeon⁷, the options available within the hospital, the individual patient and the standard of health care systems⁸. The orthopaedic literature has several references regarding non-operative treatment methods for bone non-unions. In most cases they are

based on the therapeutic application of different forms of electricity⁹⁻¹⁹, on extracorporeal shock wave therapy (ESWT)²⁰⁻²⁸, and on ultrasound²⁹⁻³².

Present clinical therapeutic approaches for the operative treatment of bone defects include bone grafts, bone transport methods (Ilizarov technique)³³, implants of different biomaterials or cell transplantation, but none of these techniques have proven to be fully satisfactory. The Ilizarov technique is based on the intrinsic regenerative potential of bone by doing an osteotomy followed by bone distraction³³. This technique aims to avoid problems with graft integration, but it is a lengthy procedure, highly inconvenient for the patient with recurrent pin track infections as a frequent complication^{34,35}. The most common method in clinical use for bone non-unions is autologous bone grafting^{4,36-38}.

Bone grafts are harvested from the patient's iliac crest and implanted into the defect. In principle these bone grafts contain all the essential components required for bone repair, such as growth factors for osteoinduction and vascularisation, cells with osteogenic potential and they provide an osteoconductive scaffold. There are, however, significant drawbacks associated with this approach. In many cases, insufficient grafts are obtained and the access to donor sites is limited^{39,40,41}. Donor site morbidity can occur and the donor bone is predisposed to fail⁴². Graft failures usually result from incomplete transplant integration, particularly in large defect sites⁴³. In addition, graft devitalisation and the subsequent resorption process can lead to decreased mechanical stability⁴⁴. Other treatment options are vascularised

autografts⁴⁵, which are surgically challenging, or the use of allogenic or xenogenic bone, which carry the risk of rejection^{46,47} and transmission of infectious disease⁴⁸⁻⁵⁰. The dense nature of cortical bone allografts impedes revascularization and cellular invasion from the host even after several months or years after implantation⁴¹. The limited ability to revascularize and remodel is believed to be the main reason for a failure rate of 25% and a 30-60% complication rate associated with allografts⁴¹.

In order to avoid the limitations associated with the current standard treatment modalities for segmental bone defects, many biomedical engineers and clinical scientists have focused their research on bone tissue engineering. For the clinical application of different novel treatment strategies, including the tissue engineering approach, an effective clinical outcome with recovery of nearly normal limb function is most important for the patient. So far, there have been no randomised clinical studies showing that a tissue engineered construct (TEC) enhances the treatment of large segmental bone defects without further application of either vascularised autografts, non-vascularised autografts, and/or allografts. In human beings, after trauma, non-union, tumour resection or chronic osteomyelitis, no standardised critical sized defects can be found that would allow a direct comparison of different reconstruction methods for bone regeneration purposes. Moreover, soft tissue conditions and therefore vascularisation issues vary from case to case.

Therefore, it is necessary to use a large preclinical animal model to have a controlled and standardised defect to evaluate the different strategies before translation into a clinical application.

1.2. Animal Models for segmental defect research

To translate research findings in the area of cell biology and biomaterials into clinical applications for bone regeneration, the use of adequate *in vivo* animal models is necessary. The first step after *in vitro* experiments is the use of small animal models *in vivo*. Advantages of experiments in small animal models are the possibility of using a large number of experimental groups due to the low costs of small animals. The application and systematic evaluation of new concepts in animal models is an essential step in the process of assessing newly developed bone grafts prior to clinical use in humans.

Several animal models have been developed over the years to verify the practicability of research approaches and stimulate clinical situations more closely. The identification and validation of an *in vivo* model is difficult as there are various differences regarding animal species (dog⁵¹, sheep⁵², pig⁵³, minipig⁵⁴, rat⁵⁵, mouse⁵⁶) and treated bone (femur⁵⁷, tibia⁵⁸, ulna⁵⁹, skull⁶⁰, mandible⁶¹). When selecting a specific animal species as a model system, a number of factors need to be considered. In comparison to humans, the chosen animal model should clearly demonstrate significant physiological and pathophysiological analogies in respect to the scientific question under investigation. Moreover, it must be manageable to operate and observe a multiplicity of study objects post-surgery over a relatively short period of time^{62,63,64}. While in the 20th century the dog model was mainly used for orthopaedic research, over the last decade the use of the sheep model increased significantly. Adult sheep offer the advantage of having a more similar body weight to humans and long bone dimensions suitable for the use

of human implants and prostheses, which is not possible in smaller species such as rabbits or smaller breeds of dogs⁶⁵. In order to ascertain whether newly developed bone graft substitutes or tissue-engineered constructs comply with the requirements of biocompatibility, mechanical stability and safety, the material must be subject to rigorous testing both *in vitro* and *in vivo*. To simulate biomechanical human *in vivo* conditions as closely as possible, and to assess the effects of implanted bone grafts and tissue-engineered constructs on segmental long bone defect regeneration, it is important that an experimental osseous injury inflicted to study bone repair mechanisms should be of the dimension to preclude spontaneous healing⁶⁶. The non-regenerative threshold of bone has been determined in research animal models inducing so-called *critical-sized* defects. Critical-sized defects are defined as “the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal⁶⁷” or as a defect which shows less than 10 percent bony regeneration during the lifetime of the animal⁶⁸. Although the minimum size that renders a defect “critical” is not well understood, it has been defined as a segmental bone deficiency of a length exceeding 2-2.5 times the diameter of the affected bone⁶⁹.

Analysing the literature on the topic of this PhD work, the published papers dealing with bone defects demonstrate inconsistencies relating to defect size, defect fixation, postoperative treatment, as well as breed, gender, age and weight of the animals^{37,70-75}. Many of the published studies have to be considered short term studies where no complete bone remodelling can be

expected during the experimental period. Moreover, the modification of commercially available internal fixation devices occurred as a reaction to implant failures early after surgery. These experimental settings do not reflect real clinical conditions. One of the most important elements in the study of segmental defect healing is the establishment of standardized methods to create reproducible test results. In previous work, the research group of Prof. Hutmacher developed and established a critical-sized tibial defect model in sheep tibiae to evaluate different tissue engineering based treatment concepts⁷⁶⁻⁷⁸.

1.3. Cellular biology

Different approaches in regenerative medicine include cell-based and scaffold-based strategies, delivery of osteoinductive growth factors, and genetic engineering.

1.3.1 Cell source

The nature of regenerative medicine will be profoundly influenced by whether the cells used are universal (allogeneic) or patient-specific (autologous). It is unlikely to be wholly one or the other that will dominate the field, however, at this early stage it is worth examining the both cells types from a scientific and translational point of view⁷⁹.

Mesenchymal stem cells (MSCs) and osteoblast cells (OB) have been studied in cell based strategies and the former is used most frequently.

Mesenchymal stem cells are multipotent progenitor cells, which have the potential to differentiate into a variety of mesenchymal tissues such as bone, cartilage, tendon, ligaments, muscle, fat and dermis^{80,81,82,83}.

These cells have shown their therapeutic capacity in several *in vitro* as well as *in vivo* studies for the regeneration of bone defects and non-unions.

1.3.2 Extracellular Microenvironment

Growth factors influence chemotaxis, differentiation, proliferation and synthetic activity of bone cells, thereby regulating physiological remodelling and fracture healing. Numerous growth factors, such as bone morphogenetic proteins (BMP), platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), and insulin-like growth factors (IGF) have a stimulating effect on bone defect healing by inducing chemotaxis, proliferation, and differentiation of osteoblasts and their precursors^{84,85}. BMP-7 has already been tested successfully in clinical studies on tibial nonunions and fibular defects^{86,87}. However, little is known about suitable combinations, concentrations, and different time points of delivery of growth factors during bone defect healing.

An easy and more physiological way of applying growth factors to bone defects is via the use of platelet rich plasma (PRP), a thrombocyte concentrate made of autologous blood. PRP is a source of autologous cytokines, chemokines and growth factors which, in the case of injury, manage and control the process of wound healing and tissue repair. The most prominent growth factors in PRP are platelet-derived growth factor

(PDGF), transforming growth factor (TGF)-1 and TGF-2, epidermal growth factor, insulin-like growth factor, basic fibroblast growth factor and vascular endothelial growth factor, which influence cell proliferation and differentiation⁸⁸⁻⁹⁰. Several previous investigations demonstrated a positive effect of PRP on wound healing^{91,92}. A vast number of studies have been performed to investigate the effect of PRP on bone defect regeneration^{89,93-96}. However, the results are ambiguous regarding the benefit of PRP to bone healing. As it is also rich in autologous fibrinogen, PRP in the liquid state can convert into fibrin clots in the solid state when activated by thrombin, similar to commercial fibrinogen. This indicates that PRP has similar functions to other hydrogels in delivering cells into 3D scaffolds⁹⁷.

1.4. Biomaterials

The limitations of current approaches necessitate the development of alternative bone repair techniques and have driven the development of scaffold-based bone engineering strategies. A suitable scaffold will possess a porous interconnected pore network (pores & pore interconnections should be at least 400 microns to allow vascularization) with surface properties which are optimized for the attachment, migration, proliferation and differentiation of cell types of interest (depending on the targeted tissue) and enable flow transport of nutrients and metabolic waste, and be biocompatible and biodegradable with a controllable rate to compliment cell/tissue growth and maturation⁹⁸. The design of these scaffolds also needs to consider physico-chemical properties, morphology and degradation kinetics. External

size and shape of the construct are of importance, particularly if the construct is customized for an individual patient. Scaffold design and fabrication via additive manufacturing has advanced tremendously over the past few years⁹⁹. The ability to create scaffolds in a layer by layer manner enables a computer animated design to be directly translated from a clinical scan (i.e. a patient CT scan) to produce customised scaffolds to fit an anatomical defect site. On implantation of a scaffold into a bone defect site, continuous cell and tissue remodelling is important for achieving stable biomechanical conditions and vascularization within the host site¹⁰⁰. Importantly, TECs should stimulate and support both the onset and the continuation of bone in-growth as well as subsequent remodelling and maturation by providing optimal stiffness and external and internal geometric shapes¹⁰¹. Scaffolds must provide sufficient initial mechanical strength and stiffness to substitute for the loss of mechanical function of the diseased, damaged or missing tissue and in addition must degrade at a rate which is compatible with new tissue in-growth and maturation^{102,103}.

It cannot be emphasized enough how essential it is to understand and control this scaffold degradation process, for successful tissue formation, remodelling, and maturation at the defect site. In the early days of tissue engineering, it was believed that scaffolds should degrade and vanish as the tissue is growing¹⁰⁴. Yet, tissue in-growth and maturation differs temporally from tissue to tissue and, furthermore, tissue in-growth does not equate to tissue maturation and remodelling. In other words a defect filled with immature tissue should not be considered “regenerated”. Hence, many

scaffold-based strategies have failed in the past as the scaffold degradation was more rapid than tissue remodelling and/or maturation¹⁰⁵.

The Hutmacher laboratory has spent the last decade translating a concept of bone tissue engineering based on slow biodegradable composite scaffolds comprising medical grade polycaprolactone (mPCL) and calcium phosphates (hydroxyapatite (HA), tricalcium phosphate (TCP)), from the *bench to the bedside*¹⁰⁶⁻¹¹⁵. After a large series of *in vitro* experiments the group consequently performed small animal studies using mice, rat and rabbit models which demonstrated the ability of composite scaffolds in combination with BMP's or cells to promote bone regeneration within ectopic sites or critically sized cranial defects¹⁰².

The scaffolds received regulatory approval from the Food and Drug Administration (USA) in 2007 and have been studied in a clinical setting in oral and maxillofacial applications (low-load bearing) for many years¹¹⁶; however at the start of the conception of the PhD thesis, scaffold-cell based concepts for orthopedic applications have been studied only in small animal models. Before translating new treatment concepts based on bone tissue engineering principles into a clinical application in orthopaedic and trauma surgery, rigorous evaluation is a *condition sine qua non* in adequate preclinical animal models.

1.5. Thesis Outline

The treatment of long bone defects and non-unions are still a major clinical as well as socio-economic problem around the globe. Beside the non-operative therapeutic options, like the application of different forms of electrical stimulation, the extracorporeal shock wave therapy, and ultrasound therapy, which are still in clinical use, several different operative treatment methods are available. There is no consensus as to the guidelines and the treatment of such defects differs a lot. Chapter II provides a review of the literature regarding clinical studies for the treatment of large bone defects and non-unions and the different treatment methods.

A number of clinicians and researchers investigate ways to treat large bone defects based on tissue engineering principles. Tissue engineering strategies for bone regeneration have great potential in regenerative medicine. But *in vitro* as well as *in vivo* studies in small and large animal models are necessary, to translate research findings into clinical use. Although, several researchers around the world have conducted different animal studies, the literature still lacks controlled studies that compare different clinical treatment strategies currently in use. The group of Prof. Hutmacher developed a standardised large animal model for the treatment of large bone defects. In Chapter III the osteogenic potential of autologous and allogenic mesenchymal progenitor cells for the reconstruction of large bone defects was analysed.

Beside mesenchymal progenitor cells (MPCs) differentiated cells (Osteoblasts) have been used in cell based tissue engineering strategies. In

previous *in vitro* and *in vivo* studies in small animal models, the differentiated cells showed a higher osteogenic potential compared to mesenchymal progenitor cells. In Chapter IV the osteogenic potential of differentiated cells were analysed for the reconstruction of critically sized large bone defects. Based on the results of the previous experiments described in Chapter III and IV the experimental setup for further experiments was changed.

In Chapter V the application method for allogenic mesenchymal progenitor cells and the scaffold design was changed and the osteogenic potential for bone regeneration was analysed.

However, based on results so far obtained in different animal models, bone tissue engineering approaches, although successful in most cases, need further validation in more clinically relevant animal models as well as in clinical pilot studies to translate bone tissue engineering approaches into clinical practice.

1.6 . Hypothesis

The limitations in solving the increasing, and somewhat difficult, orthopaedic surgery problems facing society, and the final clinical outcome for patients, may be approached from the perspective of the nature of the graft material with which the surgeon works. Current clinically established therapeutic approaches focus on the implantation of autografts and allografts, metal devices, and ceramic-based implants to assist repair of bone defects; all with inherent disadvantages. These constraints have triggered a need for new therapeutic concepts to design and engineer unparalleled structural and functioning bone grafts to replace current treatments. It is within this context that the PhD project has emerged, through the integration of engineering, life sciences, molecular and cell biology, stem cell biology, and surgery. Hence, the hypothesis of the PhD project is articulated as the following:

A validated large preclinical segmental bone defect model allows the qualitative and quantitative comparison of different scaffold-cell-based therapy concepts; it is hypothesised that osteoblasts (differentiated cells) show a higher osteogenic potential than mesenchymal progenitor cells in a scaffold based bone tissue engineering concept for regeneration of a critically sized segmental tibial defect in a large animal model.

Chapter II - Treatment of long bone defects and non-unions: from research to clinical practice

2.1. Introduction

The therapy of large bone defects and non-unions caused by trauma, revision surgery, inflammation, tumour surgery, and developmental deformity remains a major challenge for orthopaedic surgeons. There are different therapeutic options for bone non-unions and bone defects in clinical use, containing operative and non-operative treatment. The orthopaedic literature attended several references regarding non-operative treatment methods for bone non-unions. In most cases they are based on the therapeutic application of different forms of electricity, on extracorporeal shock wave therapy (ESWT) and on ultrasound^{25,29,30,117}. They led to bony consolidation even in cases of non-union after numerous failures with surgery. So far, there are no consensus guidelines and the treatment of such defects differs, depending on the surgical skills of the surgeon⁷, the options of the hospital, the individual patient, and standard of health care systems⁸. Therefore, a lot of clinicians and researchers investigate ways to treat large bone defects based on tissue engineering approaches. Tissue engineering strategies for bone regeneration present a promising option in modern regenerative medicine. The requirements for successful bone repair include an osteoinductive scaffold, growth factors to facilitate osteoinduction and

vascularisation of the graft, and cells with osteogenic potential. To translate research findings in the area of cell biology and biomaterials into clinical applications for bone regeneration, the use of adequate *in vivo* animal models is necessary. Several animal models have been developed over the years to verify the practicability of research approaches and stimulate clinical situations more closely. The identification and validation of an *in vivo* model is difficult, as there are various differences regarding animal species (dog⁵¹, sheep⁵², pig⁵³, minipig⁵⁴, rat⁵⁵, mouse⁵⁶), treated bone (femur⁵⁷, tibia⁵⁸, ulna⁵⁹, skull⁶⁰, mandible⁶¹), biomaterials under investigation (tricalciumphosphat¹¹⁸ (TCP), titanium mesh cages⁶⁹, hydroxyapatite¹¹⁹ (HA), gelatine sponge¹²⁰, poly-lactide-glycolide acid¹²¹), applied cells (autologous cells¹²², allogenic cells¹²³, xenogenic cells¹²⁴, autologous bone grafts¹²⁵) and bioactive agents like bone morphogenetic proteins¹²⁶ (BMP), platelet rich plasma¹²⁷ (PRP), bone marrow aspirates¹²⁸, growth hormones¹²⁹ (GH).

Two most recent reviews by Hutmacher's group summarizes and compares available, clinically relevant animal models to guide researchers to choose the most appropriate model^{76,130}. From this review, it can be concluded, that the literature lacks controlled studies that compare different clinical treatment strategies currently in use. Present clinical therapeutic approaches for the treatment of bone defects include bone grafts, bone transport methods (Ilizarov technique), implants of different biomaterials or cell transplantation, but none of these techniques has proven to be fully satisfactory. The Ilizarov technique is based on the bones intrinsic regeneration potential by doing an osteotomy followed by bone distraction³³. This technique aims at avoiding

problems with graft integration, but it is a long-lasting procedure, highly inconvenient for the patient with current pin track infections as a frequent complication^{34,35}. The most common method in clinical use for bone non-unions is autogenous bone grafting^{4,36-38}. Bone grafts are harvested from the patient's iliac crest and implanted into the defect. In principle these bone grafts enclose all the essentials required for bone repair, such as growth factors for osteoinduction and vascularisation, cells with osteogenic potential and they provide an osteoconductive scaffold, but this procedure is limited by the amount of bone that can be taken from the donor site and donor site morbidity^{39,40,41}. Other options are vascularised autografts⁴⁵, which are surgically challenging, or to use either allogenic bone or xenogenic bone, which carry the risk of rejection^{46,47} and transmission of infectious disease⁴⁸⁻⁵⁰. These limitations have lead scientists and clinicians to focus their research on tissue engineering aspects of bone reconstruction and developing scaffolds to support bone regeneration¹³¹.

A wide number of different biomaterials has been developed and are subject to current research. There are two major engineering approaches to develop novel treatment concepts involving biomaterials: cell-based and cell-free. In both cases, materials are engineered to provide optimal function for specific applications. In other words, scaffolds for cell-based therapies are intended to provide a compatible carrier for viable cells for enhanced histogenesis, function and integration within the recipient's tissue bed. Scaffolds for cell-free repair are designed to stimulate neo-histogenesis, often by mimicking signals for anabolic processes. In the past, different scaffolds have been

shown to be useful supports for the reconstruction of bone, however only a handful brought fully satisfactory results¹³².

For integration within the host site, solid scaffolds need to be resorbable or should have a porosity or woven form of favourable texture. Coatings and absorption or activation with bioactive molecules can also improve integration. These principles have been applied with varying success to musculoskeletal repair and reconstruction, but opportunities exist for novel procedures. When developing bone engineering strategies for the treatment of segmental bone defects, scaffolds used must be able to bear or share substantial loads immediately after implantation¹³².

Over the last years, the application of bone morphogenetic proteins (BMPs), especially BMP-2 and BMP-7, has become more important in the treatments of bone defects. These proteins belong to the Transforming Growth Factor- β (TGF- β) super family and induce osteogenic cell differentiation *in vitro*^{133,134}, *in vivo*¹³⁵ as well as bone defect healing *at the bedside*^{86,136}. The aim of this review is to summarize different operative treatment concepts for long bone non-unions and large bone defects that are currently in clinical use and to analyse and compare the different clinical studies.

2.2. Autologous bone graft

Bone grafts can be vascularised or non-vascularised. Non-vascularised bone grafts can be used alone, or in combination with internal or external fixation. Autologous bone grafts unite all the key elements, which are necessary for bone repair, such as cells with osteogenic potential, growth factors, and a

scaffold. But they are of limited supply and associated with donor site morbidity¹³⁷. The application of autologous bone grafts has advanced as the gold standard in the treatment of bone non-unions¹³⁸⁻¹⁴¹. The clinical application of bone grafts appears in the literature for the first time at the beginning of the 20th century¹⁴². In 1918 *Chutro* described a revolutionary grafting technique, which bears a remarkable similarity to today's cancellous strip grafting, except that the strips of bone were harvested from the surface of the tibia¹⁴³. Over the next years, a lot of reports were published on the biological behaviour of different types of bone graft. In 1931 *Gallie* published his early work showing the superiority of cancellous over cortical bone¹⁴⁴. *Mowlem* reached a similar conclusion in a review of 75 cases in which cancellous bone had been used to treat bone defects of the skull, the mandible and in long bones¹⁴⁵. Higgs treated 60 cases of non-union of long bones with cortical grafts in 20 cases and with cancellous bone chips in 40 cases¹⁴⁶. All achieved bone union, but the time to union was less, when cancellous chips compared to cortical bone were used. In a review of 100 cases of non-unions of *Sakaellarides et al.*¹⁴⁷ in 1964, autologous bone grafts were used to treat bone non-unions. In 56 cases, uninfected non-unions were treated by autologous bone-grafting from the iliac crest (35 cases) or a cortical onlay graft (21 cases) and achieved bone union in 46 cases (82%). Seven cases required revision surgery and only three did not achieve bone union (3.5%). The authors came to the conclusion that if there is some stability, the transplantation of cancellous iliac bone seems to be one of the best methods of treatment. In 1969, *Souter* published his experiences of 102 cases of delayed union and non-union of long bones¹⁴⁸. Cancellous strip

grafting from the iliac crest alone were used in 68 cases and 34 of them needed additionally an excision of the fibrous tissue, correction of misalignment and internal fixation of the fragments with a plate. The average time to bone union varied from 10-16 weeks. Only four grafts out of 102 cases failed to consolidate within 16 weeks. *Reckling and Waters* treated 44 cases of tibial non-unions with cancellous bone grafts from the iliac crest¹⁴⁹. Bone union was obtained in 97,7%, whereas three patients needed two bone-grafting procedures. Similarly results were described by *Gershuni and Pinsker*, who treated 40 tibial non-unions with autologous bone grafts and achieved union in 85% after 20 weeks¹⁵⁰.

Open intramedullary nailing and bone grafting with either autologous cancellous bone grafts from the iliac crest or cortical reamings of bone from the intramedullary canal were compared by *Johnson and Marder* in 22 tibial non unions¹⁵¹. All cases went to union (100%). Compared with closed techniques, the authors identified a reduction of time to healing. Another important difference between these methods is the ability to augment the site of non-union with bone grafts and the ability to remove the scar tissue, to obtain anatomical correction of the fragments. *Sledge et al.* retrospectively analysed the records of 51 patients who were treated with intramedullary nailing and reaming for non-union of the tibia¹⁵². In 10 cases autologous bone grafts from the iliac crest were used. In 49 (96%) of the 51 patients, tibial union occurred at an average of seven months postoperatively. No statistically significant difference was found between open or closed intramedullary nailing techniques and the use of autologous bone grafts from

the iliac crest for enhancement. This study demonstrated that nailing with reaming is a good treatment with acceptable rates of union also predicated from other recent reports on the use of nailing with reaming for non-union^{151,153,154}. There were certain clinical situations in which intramedullary nailing techniques have been problematically, because the non-union is associated with a number of external skeletal fixations^{152,155,156}, remote infections¹⁵⁷⁻¹⁶⁰, or deformity¹⁶¹⁻¹⁶³. *Helfet et al.* treated 33 patients who had a tibial non-union with limited open exposure, indirect reduction with a femoral distracter, tension band plating, lag screw fixation, and autologous bone grafting¹⁶⁴. All the non-unions healed at an average of four months. In terms of complications, four superficial skin breakdowns, one deep infection, and one fracture of the plate were reported. The authors came to the conclusion, that this plating technique is an acceptable alternative for the treatment of aseptic non-unions of the tibial diaphysis, for which standard locked intramedullary nailing is contraindicated. *Wiss et al.* published in 1992 a prospective non-randomised study of 39 cases of tibial non-unions, treated with autologous bone grafting¹⁶⁵. After 7 months postoperatively, union rates of 96% have been achieved on average. A retrospective comparative analysis of different biological enhancement options for persisting tibial non-unions was published in 1995 by *Brighton et al.*¹⁶⁶. The authors compared a large number of difficult cases of non-unions with direct currents or bone graft surgery. They had a number of 171 aseptic non-unions, of which 33 were treated with capacity coupling (20 microampere of constant direct current), 114 with electric currents (60-kilo-hertz, 5-volt peak-to-peak symmetrical sine wave signal) and 24 with autologous bone graft from the iliac crest. Of the

cases treated with bone grafting, only 58.3% healed. Thus, especially in difficult cases where previous grafting had failed, the failure rate of a secondary bone grafting was much higher. The tibia is the long bone with the highest incidence of non-union because of its poor soft tissue coverage and it is also the most common long bone to sustain a fracture. In a review article on tibial non-union treatment options, *Wiss et al.* commented that autologous cancellous grafts remains the gold standard in the treatment of tibial non-union and can be used to augment segments up to 6cm in space with a successful outcome in 88% to 95% of all cases.

In 2001, *Friedlaender et al.*⁸⁶ reported their experiences with 124 tibial non-unions in a multi-centre clinical trial. They treated 61 cases of non-unions with autologous bone grafts and showed clinical success with full weight bearing in 85%. After 12 months, 13% of these patients still had persistent pain at the donor site. The use of autologous bone grafts for non-union is also described in several other long bones. In the series of *Devnani* 25 non-unions were treated with compression plate fixation and autologous bone grafts¹⁶⁷. The non-unions involved seven femora, eight humerus and ten tibiae. All non-unions healed on average within 18 weeks. One femoral plate broke after twelve weeks, which required replating. The author came to the conclusion, that plate fixation is a useful and effective means in the management of non-unions and comparable with other methods. *Ring et al.* described the use of autologous bone grafts in 35 patients with forearm non-unions¹⁶⁸. All of the non-unions healed within six months without the need for subsequent procedures. *Barbieri et al.* reported their experiences on twelve

patients with forearm non-unions treated with iliac crest bone block grafting¹⁶⁹. Healing was achieved in 83.3% without additional procedures. In another retrospective study, *Hierholzer et al.* reported their results of the treatment of 45 aseptic non-unions of the humerus¹⁷⁰. After an average of 18 weeks, 100% achieved bony union. *Hsu et al.* performed a retrospective review of 105 humeral non-unions¹⁷¹. The treatment included open reduction, plate fixation and the application of autologous bone grafts. All cases achieved union after an average of 16 weeks. *Lin et al.* reviewed retrospectively 86 patients with aseptic non-unions of the humeral shaft¹⁷². All non-unions were treated with removal of the previous implant, open reduction, and internal fixation supplemented by cancellous bone grafts. All of the non-unions united within an average time of 18 weeks. The femur has one of the best healing rates in the body. Therefore, reports on femoral non-unions are rare and often involve only small sample sizes. *Chapman and Finkemeier* reported a retrospective analyse of 18 cases of femoral non-unions, all treated with internal fixation and autologous bone grafting¹⁷³. The same authors reviewed a series of 39 patients with femoral non-unions, treated with reamed intramedullary nailing¹⁷⁴. They had bony unions after an average of 19 months in all cases. They recommended reamed exchange nailing for the treatment of femoral non-unions prior to bone grafting reserving autologous bone grafting as a second line procedure.

Most recently, the Reamer-Irrigator-Aspirator (RIA) was developed as an alternative method for long bone canal reaming in the process of intramedullary nailing to reduce the incidence of fat embolism and thermal

necrosis. Particles aspirated by the RIA are caught in a coarse filter, from which they can be recovered and used as a source of autologous bone grafts. It has been shown, that the bony reaming debris as well as the irrigation fluid is a rich source of growth factors¹⁷⁵ and a source of mesenchymal stem cells¹⁷⁶, which are able to grow, proliferate and differentiate into the osteogenic pathway in vitro. Large volumes of graft can be obtained, with up to 90ml harvest being reported in different clinical studies and case series (Fig. 1). *Belthur et al.* showed that using RIA derived grafts (37 of 41 cases) compared well with an iliac crest bone graft control group (32 of 40 cases). *Miller et al.* reported a series of 13 patients treated with RIA bone grafting enhanced with Dexamethasone. All patients displayed at least partial union at most recent follow up, but only six patients went on to complete union. All patients treated had persisting non-union or significant bone defects that failed to heal with standard procedures. The obvious limitation of several studies is that it is really a collection of case reports or case series, with different procedures and surgical techniques.

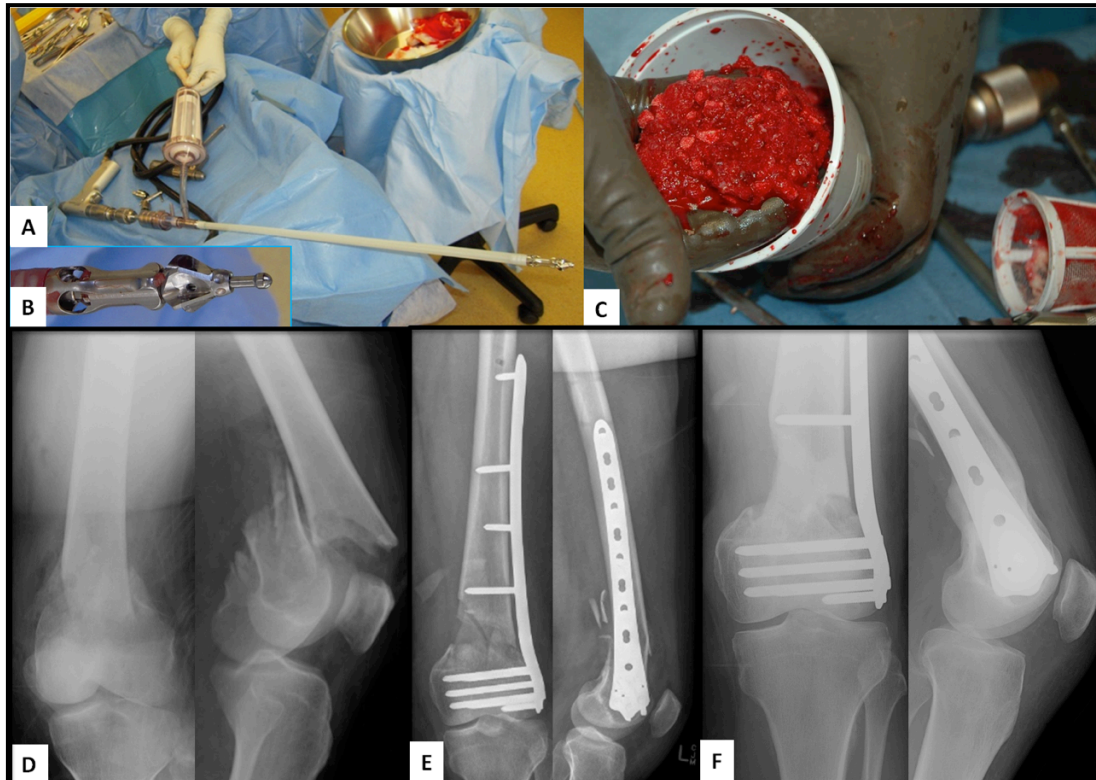


Figure 1: Collection of autologous osseous particles mixed with bone marrow (B), by using the reamer-irrigator-aspirator (RIA) system (A). The drill head of the system applies irrigation and aspiration to the medullary canal. An open distal femur fracture of an 32-year old male patient after an motor bike accident was stabilized using a LISS-plate (D). The radiographic images after surgery showed a remaining bone defect, which were treated in a second operation with autologous bone particles and bone marrow obtained via intramedullary reaming by using the RIA system (E). The follow up radiographic images after 6 months showed bony union (F).

Author	Year	Number of non unions	Bone	Treatment	Average time to union (in weeks)	Rate of union
Higgs ¹⁴⁶	1945	60	Tibia	ABG/CBG	--	100%
Sakellaridis et al. ¹⁴⁷	1964	56	Tibia	ABG/CBG	34,8/48,4	89%/71%
Souter et al. ¹⁴⁸	1969	102	Tibia/Forearm/Humerus/Femur	ABG	16	96,6%
Reckling and Waters ¹⁴⁹	1980	44	Tibia	ABG	30	97,7%
Gershuni and Pinsker ¹⁵⁰	1982	40	Tibia	ABG	20	85%
Johnson and Marder ¹⁵¹	1987	22	Tibia	ABG/RBP	12,5	100%
Sledge et al. ¹⁵²	1989	51	Tibia	RBP/ABG	28	96%
Simon et al. ¹⁷⁷	1990	30	Tibia	ABG	--	97%
Wiss et al. ¹⁶⁵	1992	39	Tibia	ABG	28	96%
Helfet et al. ¹⁶⁴	1992	33	Tibia	ABG	16	100%
Brighton et al. ¹⁶⁶	1995	24	Tibia	ABG	40	58,3%
Barbieri et al. ¹⁶⁹	1997	12	Forearm	ABG	--	83,3%
Chapman and Finkemeier ¹⁷³	1999	18	Femur	ABG	--	100%
Devnani ¹⁶⁷	2001	25	Tibia/Humerus/Femur	ABG	18	100%
Friedlaender et al. ⁸⁶	2001	61	Tibia	ABG	36	85%
Finkemeier and Chapman ¹⁷⁴	2002	39	Femur	RBP	76	100%
Ring et al. ¹⁶⁸	2004	35	Forearm	ABG	24	100%
Hsu et al. ¹⁷¹	2005	105	Humerus	ABG	16	100%
Hierholzer et al. ¹⁷⁰	2006	45	Humerus	ABG	18	100%
Belthur et al. ¹⁷⁸	2008	81	Humerus, Femur, Tibia, Ankle	ABG (40x) RIA (41x)	--	80% (ABG) 90% (RIA)
Lin et al. ¹⁷²	2009	86	Humerus	ABG	18	100%
Miller et al.	2010	13	Humerus, Femur, Tibia	RIA	--	46%

Table 1: Clinical application and outcomes of the treatment of non-unions with autologous bone grafts (ABG), reamer-irrigator aspirator method (RIA), cortical bone grafts (CBG) or reamed intramedullary nailing (RBP).

2.3. Distraction osteogenesis and vascularised bone grafts

Vascularised bone grafts have been proposed to have significant advantages over conventional non-vascularised grafts¹⁷⁹. These vascularised bone grafts

can be taken from various bones, such as the fibula¹⁸⁰⁻¹⁸², in varying compositions, including skin, fascia or muscle¹⁸³, as a vascularised solid iliac crest bone graft¹⁸⁴⁻¹⁸⁶, the lateral border of the scapula¹⁸⁷⁻¹⁸⁹, or vascularised rib grafts¹⁹⁰⁻¹⁹². The disadvantages of these vascularised grafts are the need for microsurgical skills^{193,194}, the possibility of total transplant necrosis due to anastomotic complications¹⁹⁵, and donor site morbidity¹⁹⁶. Distraction osteogenesis, which was established by *Ilizarov*¹⁹⁷⁻²⁰⁰ in the middle of the last century, is another approach to repair large segmental bone defects. It is therefore often described as the “Ilizarov technique”. The advantages are the possibility to correct deformities²⁰¹, small areas of soft tissue defects, and immediate mobilization. Its disadvantages are the long duration of external osteosynthesis materials, frequency of pin tract infection, and pain accompanying the transport²⁰². Several surgeons reported their experiences with the Ilizarov technique and free vascularised grafts in a series of case reports, but comparative studies between the different treatment strategies are rare. Free vascularised fibular grafts were first described in the late 1970’s by *Taylor et al.*, who used this technique in two cases with extensive bone and skin loss⁴⁸. The technique was developed to salvage two legs, which would otherwise have been amputated. A few years later, *Weiland et al.* described their experiences with this technique in 32 cases¹⁷⁹. They performed 22 free vascularised fibular grafts in the lower extremity and ten cases in an upper extremity. The defects were caused either by trauma or tumour surgery. The average length of bone defects was 14.9 cm in the tumour cases and 16.2 cm for trauma cases. In 12.5% the operation was unsuccessful, resulting in amputation.

In 1989, *Paley et al.* published their results of 25 patients, treated with the Ilizarov method²⁰³. The tibial bone defects had an average size of 6.2 cm. Bone union was achieved in all cases within an average time of 54.4 weeks. *Golyakhovsky and Frankel* treated eleven patients with large tibial (9), femoral (1), and humeral (1) bone defects with the Ilizarov method²⁰⁴. They achieved bone union in all cases with excellent results in eight cases. *Green* compared the non-vascularised bone graft technique with the segmental bone transport technique for the treatment of skeletal defects²⁰⁵. Fifteen patients with skeletal defects were treated with non-vascularised bone grafts and 17 patients with callus distraction. Bone union was achieved in 86.6% of the bone graft group and 94.1% in the callus distraction group. Surprisingly, the treatment time compared to the defect size was identical in both groups: 1.9 months per centimetre of defect. The authors came to the conclusion, that short defects (up to 5 cm) could be treated with either segmental bone transport or cancellous bone grafts, depending on the surgeon's preferences. Whereas large gaps in bone tissue require a more complex reconstructive technique, either bone transport or a free vascularised bone graft. *Suger et al.* treated 20 patients with bone and soft tissue defects by open segmental bone transport according to Ilizarov²⁰⁶. In 19 cases the bone defect could be filled by callus distraction only, while one patient needed additional cancellous bone transplantation. *Polyzois et al.* had experiences with the Ilizarov technique in tibial and femoral bone loss in 28 infected and 14 aseptic cases²⁰⁷. These 42 patients with diaphyseal bone defects (25 tibial and 17 femoral) were treated by radical debridement and bone transport. The

average defect size was 6 cm. Union was achieved in 38 patients after an average treatment period of 40 weeks. *Oztürkmen et al.* treated 46 patients with pseudarthrosis of the femur (n=8) and the tibia (n=38) with the Ilizarov method²⁰⁸. The mean defect size was 5.2 cm. Union occurred in 42 (92%) cases. The mean time for the fixator appliance was 208 days. Pin tract infections occurred in 28 cases. *Kocaoglu et al.* used the callus distraction over an intramedullary nail combined with an external fixator for the treatment of 13 patients with an average bone defect size of 10 cm²⁰⁹. Seven patients had a segmental tibial defect and six patients a segmental femoral defect. In two patients a free non-vascularised fibular graft was added to the distraction site for augmentation of a femoral defect at the time of external fixator removal. Eleven patients showed excellent results after a mean follow-up of 47 months, in two cases the nail had to be removed because of an infection. The patients underwent revision with an Ilizarov fixator and the non-unions healed. The main time for the use of an external fixator was 19.3 weeks. This combined method allowed an earlier removal of the external fixator associated with an increased patient comfort, a decreased complication rate, and a convenient and rapid rehabilitation. *Yokoyama et al.* treated four patients with tibial bone defects with free vascularised fibular grafts and another four patients with distraction osteogenesis²¹⁰. The mean defect length in the vascularised bone graft group was 7.3 cm and in the callus distraction group 4.6 cm. The union rate of the callus distraction group was 100%, whereas the vascularised graft group showed a union rate of 75%. The authors assessed to the costs associated with both treatments. No clear

differences could be determined between the two treatment groups, regarding costs and functional outcomes.

Morasiewicz et al. reported their experiences with the Ilizarov method in 16 cases of femur non-unions²¹¹. In a first operation the authors tried to reach bone union by femur shortening. In the second and the third operation the authors tried to correct the femoral axis and to re-establish the length of the affected bone. In 93.75% bone union was achieved, just one case resulted in pseudarthrosis. *Zarek and Macias* used the Ilizarov method for the treatment of pseudarthrosis of the humerus in 20 cases²¹². The mean time of treatment with the external fixator was 29 weeks and the main complication during treatment was superficial pin-tract infection (in 40%). Bone union was achieved in 19 cases (95%). In all clinical studies, the authors came to conclusion, that the Ilizarov method is a simple and safe method for successful treatment of diaphyseal bone defects. The main disadvantage of the Ilizarov method is the long external fixation time and pin-tract infections. If a large soft tissue defect needs also to be treated, the free vascularised bone graft with muscle and skin tissue might have advantages.

To overcome the problems with pin-tract infections and the discomfort for the patient by using an external fixateur, motorised, fully implantable, solid intramedullary nails have been developed in the 1990s^{213,214}.

Author	Year	No. of patients	Bone	Treatment	Time of treatment with external fixator (in weeks)	Size of Defect (average in cm)	Rate of union
Weiland et al. ¹⁷⁹	1983	32	Tibia/Humerus	FVFG	--	15.8	87.5%
Paley et al. ²⁰³	1989	25	Tibia	CD	--	6.2	100%
Golyakhovsky and Frankel ²⁰⁴	1991	11	Tibia/Femur/Humerus	CD	48	4-16	100%
Green ²⁰⁵	1994	32	Skeletal defect	15 ABG/ 17 CD	ABG 31.6/ CD 38.4	ABG 4/CD 5	ABG 86.6% CD 94.1%
Suger et al. ²⁰⁶	1995	20	Skeletal defect	CD	--	--	95%
Polyzois et al. ²⁰⁷	1997	42	Tibia/Femur	CD	40	6	90.5%
Yokohama et al. ²¹⁰	2001	8	Tibia	4 CD/4 FVFG	CD 37.3/ FVFG 25.1	CD 4.6/FVFG 7.3	100%/75%
Zarek and Macias ²¹²	2002	20	Humerus	CD	29	--	95%
Oztürkmen et al. ²⁰⁸	2003	46	Tibia/Femur	CD	29.7	5.2	92%
Kocaoglu et al. ²⁰⁹	2006	13	Tibia/Femur	CD+IMN	19.3	10	84.6%
Morasiewicz et al. ²¹¹	2007	16	Femur	CD	--	--	93.75%

Table 2: Clinical application and outcomes of the treatment of bone defects and bone non-unions with callus distraction “Ilizarov technique” (CD), free vascularised fibular grafts (FVFG), intramedullary nailing (IMN), and autologous bone graft (ABG).

2.4. Tissue Engineering

According to Langer and Vacanti, tissue engineering is “an interdisciplinary field that applies the principles of engineering and life science towards the development of biological substitutes that restore, maintain, or improve tissue function”²¹⁵. Over the last two decades, a small group of researchers have

focused on approaches for the reconstruction of segmental bone defects or bone non-unions. Many research groups around the world aim to find suitable combinations of biomaterials, cells with osteogenic potential and growth factors for the therapy of bone defects or bone non-unions that allow an functional tissue engineering approach.

2.4.1. Growth factor delivery

Growth factors, especially BMPs, seem to play an import role in osteogenic differentiation. In 1965, *Urist's*²¹⁶ discovery of the osteoinductive capacity of demineralised bone matrix brought attention to the role of growth factors found within. In later years, more than 15 osteogenic proteins or bone morphogenetic proteins (BMP) were discovered. Each of these proteins play a specific agonistic and/or antagonistic action in the progressive stages of bone callus formation^{217,218}. Their osteoinductive potential was shown in several *in vitro* and *in vivo* studies²¹⁹⁻²²². Based on these *in vitro* and *in vivo* findings, bone morphogenetic proteins (BMP-7 and BMP-2) were produced by recombinant DNA technology for clinical application²²³. *Friedlaender et al.* started a randomized multicenter study in 1992, including 122 patients with 124 tibial non-unions, that was published in 2001⁸⁶. The fixation method for all patients was intramedullary nailing in combination with the local application of rhBMP-7 (recombinant human bone morphogenetic protein-7 or OP-1) in a type I collagen carrier (3.5 mg of rhOP-1 mixed with 1g of type I bovine bone-derived collagen) in 63 non-unions or the application of autologous bone grafts in 61 non-unions. At nine months following the surgical procedure, 81% of the OP-1 treated non-unions and 85% of those

receiving autogenous bone were assessed by clinical criteria to have been treated successfully. By radiographic criteria, at the same point, 75% of those in the OP-1 treated group and 84% of the autograft treated group showed bony unions. Overall, the OP-1 administration was safe and proved to be statistically comparable to the gold standard biologic enhancement of autograft. This randomized trial of *Friedlaender et al.*⁸⁶ have established BMP's as a bone graft option. The efficacy of recombinant human bone morphogenetic protein-2 (rhBMP-2), as an adjunct to the standard care of the primary treatment of open tibial fractures, has been investigated in the BMP-2 Evaluation in Surgery for Tibial Trauma (BESTT) study¹³⁶. In total, 450 patients with open tibial fractures were randomized to receive the standard of care (intramedullary nail fixation and routine soft-tissue management), the standard of care and an implant containing 0,75 mg/ml of rhBMP-2 (total dose of 6 mg, solved in 8 ml of sterile water), or the standard of care and an implant containing 1.50 mg/ml of rhBMP-2 (total dose of 12 mg, solved in 8 ml of sterile water). The rhBMP-2 implant was placed over the fracture at the time of definitive wound closure. A significant reduction in the risk of secondary intervention was observed in the 1.50 mg/ml rhBMP-2 group compared with the standard of care alone group. These results suggested that rhBMP-2 is an alternative to the standard of care alone in patients with acute open tibial fractures. Whereas *Aro et al.*²²⁴ showed no differences in the treatment of open fractures with BMP-2. Both groups were treated with reamed nails. There was no difference in healing between standard of care and BMP, but an increased infection rate in the BMP-2 group. Another research group identified the presence of endogenous BMP, their receptors

and mediators in a non-union microenvironment²¹⁷. In their immunohistochemical analysis of samples from 17 non-unions and four delayed unions they proved the presence of these molecules in 81% of the samples, probably at suboptimal levels²²⁵ relative to those necessary for adequate osteogenesis and bone union.

In the 80's, *Johnson et al.* treated six patients with traumatic segmental tibial bone defects with autogenous cancellous bone grafts combined with human BMP and other insoluble noncollagenous proteins (hBMP/iNCP)²²⁶. The authors did not describe the type of BMP and the exact concentration. The tibial defects ranged from 3-17 cm. The hBMP/iNCP was suspended in a chloroform solution of polylactic acid/polyglycolic acid copolymer and poured into a Teflon mold 0.2 x 2.0 x 13 cm in length. This strip was placed around the defect, whereas the autologous bone grafts filled the intercalary defect. Five of the six defects healed without further surgical treatment. From 1989 to 1998 the same author treated 30 patients with femoral non-unions with an allogenic autolysed antigen-free allograft (AAA) and 100 mg of partially purified human Bone Morphogenetic Protein (hBMP) lyophilized into the implant²²⁷. The fixation was achieved with a 95° fixed angle plate in 25 cases, intramedullary nailing in three cases, and in two cases with a condylar buttress plate. Thirteen of 30 patients received additional autogenous bone grafts to the intercalary segmental defect. Twenty-four (80%) of the femoral non-unions healed, whereas six patients had fatigue failure of the plate implant. *Dimitriou et al.* evaluated the efficacy and safety of rhBMP-7 (OP-1) as a bone-stimulating agent in the treatment in of 25 patients with 26 non-

unions²²⁸. There were 10 tibial non-unions, eight femoral, three humeral, three ulna, one patella, and one clavicle non-union. In 17 cases the application was combined with autologous bone grafting. In 24 cases (92.3%) clinical and radiological union occurred within a mean time of 4.2 months. One case was a complicated fracture with an infected non-union and with multiple previous limb salvation procedures and underwent a below knee amputation. *Ronga et al.* reported in an observational, retrospective, non-randomized study the use of BMP-7 for the treatment of non-union's in various anatomic sites²²⁹. The work was performed by the BMP-7 Italian Observational Study (BIOS) Group. The clinical series included 105 patients. Additional grafts were used based on the surgeon's decision. Radiographic and clinical assessments were carried out at progressive time intervals on two main groups: BMP-7 + autograft (n=50) or BMP-7 (n=38). The authors did not describe the exact concentration of BMP. In eleven cases they implanted BMP-7 with an osteoconductive agent and in six cases with a composite graft (bone marrow, autologous growth factors, allograft, hydroxyapatite, fibrin glue). The mean follow-up was 29.2 months. The cases treated with a composite graft were excluded, because they were not comparable due to the complexity of the treatment. At nine months there was an overlapping between the unions recorded in the two main groups with healing rates of 86.0% and 85.7%, respectively. The authors came to the conclusion, that this is an observational study that illustrates the efficacy of BMP-7 with and without bone grafting for the treatment of long bone non-unions. In 2008, *Kanakaris et al.*²³⁰ published their multicenter study focused on the application of BMP-7/OP-1 for the treatment of aseptic tibia non-

union's. Sixty-eight patients fulfilled the inclusion criteria for this observational study, with a minimum follow-up of twelve months. In 41% the application of BMP-7 was combined with revision of the fixation at the non-union site. In 25 cases the BMP-7 implantation was augmented by autologous bone grafts. Non-union healing was verified in 61 (89.7%) within a median period of 6.5 months (range 3–15 months). No adverse events or complications were associated with BMP-7 application. The safety and efficacy of BMP-7 was verified in this case series, and was comparable to previously published study results. *Giannoudis et al.* explored the synergistic effect of autografts and BMP-7 in the treatment of atrophic non-unions²³¹. In total, 45 patients with seven humeral, 19 femoral, and 19 tibial non-unions were included in this study. All non-unions were atrophic and seven had bone defects of a median length of 2.5 cm (2-4 cm). All of the 45 patients progressed to non-union healing (100%). *Moghaddam et al.* published their experiences with 54 patients who had atrophic non-unions of long bone fractures²³². The localization of the non-unions included 21 in the femur, 26 in the tibia, three in the humerus and seven in the forearm. In 36 cases, BMP 7 was used in combination with osteosynthesis revision and bone grafting; in 9 additional patients, BMP-7 was used with bone grafting alone. In twelve patients, BMP-7 was applied as a single procedure without any bone grafting or any change in osteosynthesis. Three patients with a non-union in two different long bones received one unit of BMP-7 at each bone site (3.5 mg). Forty-seven of the 57 (82%) implantations were successful, with bony healing confirmed by clinical and radiological evaluations. *Alt et al.* performed a study to analyse the cost savings from a societal perspective for the use of BMP-2 in the treatment of

open tibial fractures (grade III A and III B) in the UK, Germany, and France⁸. Health care system costs (direct health care costs) and costs for productivity losses (indirect health care costs) were calculated using the raw data from the Bone Morphogenetic Protein Evaluation Group in Surgery for Tibial Trauma “BESTT study”. Overall treatment costs per patient and year after the initial surgery of the control vs. the rhBMP-2 group were compared. In summary, despite the apparent high direct costs of rhBMP-2 in the treatment of grade III A and B open tibial fractures, indirect net cost savings were calculated for all three countries.

Most of the mentioned studies have limitations, as no randomisation was performed, no control groups were used or the number of patients included was too small. Nevertheless, the clinical application of the commercially available BMP-2 and BMP-7 seems to be a safe procedure for the patient without any major complications in any of the mentioned studies. But long-term effects are still not explored. The clinical application of BMP showed a very good healing rate (80-100%), even in cases with several unsuccessful previous operations, but even in some cases, BMP cannot lead to a successful treatment (Fig. 2). Because of the outcome of the BMP treatment, even in therapy resistant non-unions, BMP should be perceived as a therapeutic option that, given specific indications, should not be denied to patients.

Author	Year	No. of patients	Bone	Biological enhancement	Size of Defect (average in cm)	Rate of union
Johnson et al. ²²⁶	1983	6	Tibia	BMP-7 + ABG	8.3	83.3%
Johnson and Urist ²²⁷	2000	30	Femur	hBMP/AAA 13x add. ABG	2.7	80%
Friedlaender et al. ⁸⁶	2001	122	Tibia	OP-1 63, ABG 61	--	OP-1 81%, ABG 85%
Dimitriou et al. ²²⁸	2005	26	Tibia, Femur, Ulna, Clavicle	BMP-7 17x add. ABG	--	92.3%
Ronga et al. ²²⁹	2006	105	Tibia, Femur, Forearm Humerus, Clavicle	38xonly BMP-7 --- 11xwith add. OA -50xwith add. ABG - 6xwith CG	--	BMP 86,8% BMP+ABG 86% BMP+OA 85,7%
Kanakaris et al. ²³⁰	2008	68	Tibia	43xBMP-7 25xBMP+ABG	--	89.7%
Giannoudis et al. ²³¹	2009	45	Tibia, Femur, Humerus	BMP-7+ABG	2.5 (n=7)	100%
Maghaddam et al. ²³²	2009	54	Femur, Tibia, Humerus, Forearm	36xBMP-7+OR 9xBMP-7+ABG 12xBMP-7	<1,5	82%

Table 3: Clinical application and outcomes of the treatment of bone defects and bone non-unions with revision osteosynthesis (intramedullary nailing (IMN), plate osteosynthesis (PO) and external fixation (EF)) in combination with biological enhancement such as bone morphogenetic protein 7 (BMP-7 or rhOP-1), autologous bone grafts (ABG), composite grafts (CG, as bone marrow, autologous growth factors, allograft, hydroxyapatite, fibrin glue), osteoconductive agents (OA), (add.=additional),(--=no data available).



Fig. 2: A femur non-union of a 79-year old patient after plate osteosynthesis. The patient had a sarcoma of the thigh with radiation as a treatment. Afterwards, he fell at home and broke his femur. The femur non-union was treated with an cortical bone chip, fixed with two additional screws (A). After 6 months, no healing occurred, and application of cancellous bone in combination with growth factors (BMP-7) was performed (B). After another 4 months, implant failure occurred without any progress in the bone regeneration (C). Therefore, the final decision was made to implant a prosthesis (D), which was still in place with a good limb function of the patient after 1 year (E).

2.4.2. Cell based strategies

Other approaches in regenerative medicine are cell-based strategies for the reconstruction of bone defects. Mesenchymal stem cells, periosteal cells and osteoblasts have been employed in these cell-based strategies. Among these cells, mesenchymal stem cells are used most frequently, and seem to be a potential cell source for regeneration of mesenchymal tissues such as bone. They are multipotent progenitor cells, which have the potential to differentiate into a variety of mesenchymal tissues such as bone, cartilage, tendon, ligaments, muscle, fat and dermis^{80,81,82,83}. The cells can be isolated from a variety of tissues^{233,234,235}, such as bone, bone marrow, fat and periosteum, using different separation techniques and may be differentiated into the appropriate phenotype under defined culture conditions and the influence of specific growth factors or cytokines²³⁶. Their advantages are unlimited supply and easy accessibility by bone marrow aspiration, the possibility of *in vitro* expansion of the cells and a high osteogenic potential, which has been extensively studied. In contrast to the little knowledge about the use of mesenchymal stem cells for the repair of skeletal lesions in humans, they have been studied extensively in several *in vitro* studies²³⁷⁻²⁴⁰, as well as *in vivo* studies in different animal models²⁴¹⁻²⁴⁴ and have demonstrated their bone-forming capacity and usefulness in treating critical size bone defects. Although strong efforts have been made over the last decade to introduce stem cell and tissue engineering treatment strategies to the field of orthopaedics, only a few clinical applications are currently available.

Based on results of experiments regarding the reconstruction of large bone defects in large animal models²⁴⁵, *Quarto et al.* were the first to report the repair of extensive bone defects (4-7cm) in humans by means of implantation of a porous ceramic scaffold seeded with *in vitro* expanded bone marrow derived autologous mesenchymal stem cells²⁴⁶. Three patients were selected for this cell-based tissue engineering approach after failure of alternative surgical therapies. For each patient, osteoprogenitor cells were isolated from bone marrow and expanded *ex vivo*. These cells were placed on macroporous hydroxyapatite scaffolds, the size and shape of which reflected the particular bone defect in each patient, and implanted at the lesion sites. External fixation was provided initially for mechanical stability and was subsequently removed (6.5 months after surgery in Patient 1, 6 months after surgery in Patient 2, and 13 months after surgery in Patient 3). In all three patients, radiographs and computed tomography scans revealed abundant callus formation along the implants and good integration at the interfaces with the host bones by the second month after surgery. All three patient recovered limb function within six to twelve months. Later they included one more patient in their study and made a follow up after six to seven years²⁴⁷. In all patients a good integration of the implants was maintained. *Vacanti et al.* reported the case of a man who had a traumatic avulsion of the distal phalanx of the thumb²⁴⁸. The bone was replaced with *in vitro* expanded autologous periosteal cells that had been expanded and seeded onto a porous coral implant. Twenty-eight monthss after the implantation, the patient had a thumb of normal length and strength. *Kitoh et al.* reported the clinical results of distraction osteogenesis with transplantation of bone marrow-

derived mesenchymal stem cells (BMSCs) and platelet-rich plasma (PRP) in 24 bones (12 femora and 12 tibiae) of 20 patients with achondroplasia and hypochondroplasia²⁴⁹. BMSCs derived from the iliac crest were cultured with osteogenic supplements and differentiated into osteoblast-like cells. Culture-expanded osteoblast-like cells and autologous PRP were injected into the distracted callus with a thrombin–calcium mixture so that the PRP gel might develop within the injected site. Transplantation of BMSCs and PRP was done at the lengthening and in 5 patients additionally at the consolidation period. The target lengths were obtained in every leg without major complications. Surprisingly, the average healing index of the BMC-PRP group (27.1 +/- 6.89 d/cm) was significantly lower than that of the control group (36.2 T 10.4 d/cm), (p = 0.0005). But the femoral lengthening showed significantly faster healing than did the tibial lengthening in the BMC-PRP group (p = 0.0092). However, the contribution of the injected cells to the bone formation is difficult to evaluate due to the inconsistent number of cells injected and the different ways of the cell application. Furthermore, the author's conclusion that the transplantation of cells with PRP shortened the treatment period of the distraction osteogenesis cannot be accepted and comprehended. *Morishita et al.* reported the treatment of bone defects caused by bone tumours in three patients using tissue-engineered implants²⁵⁰. The number of mesenchymal stem cells (MSCs) obtained from each patient's bone marrow cells was first increased and the MSCs were forced to differentiate into osteoblasts followed by bone matrix formation on hydroxyapatite (HA) ceramics. The tissue-engineered HA was used to fill the patient's bone cavity after tumour curettage. The authors describe, that an

immediate healing potential was found by serial plain radiographs and computed tomography images, and no adverse reactions were noted in these patients. These results indicate that tissue-engineered osteogenic ceramics might be an alternative to autologous bone grafts. The percutaneous use of autologous bone-marrow cells for the treatment of non-unions was performed by *Hernigou et al*²⁵¹. The purpose of his study was to evaluate the number and concentration of progenitor cells that were transplanted for the treatment of non-union, the callus volume obtained after the transplantation, and the clinical healing rate. Bone-marrow was aspirated from both anterior iliac crests, concentrated on a cell separator, and then injected into 60 non-infected atrophic non-unions of the tibia. Each non-union received a relatively constant volume of 20 cm³ of concentrated bone marrow. An average total of 51×10^3 fibroblast colony-forming units were injected into each non-union. Bone union was obtained in 53 patients, and the bone marrow that had been injected into the non-unions of those patients contained >1500 progenitors/cm³ and an average total of $54,962 \pm 17,431$ progenitors. The concentration (634 ± 187 progenitors/cm³) and the total number ($19,324 \pm 6843$) of progenitors injected into the non-union sites of the seven patients in whom bone union was not obtained were both significantly lower ($p = 0.001$ and $p < 0.01$, respectively) than those in the patients who obtained bone union. The efficacy of percutaneously autologous bone-marrow grafting seems to be an effective and safe method for the treatment of bone non-unions. *Jäger et al.* used bone marrow concentrate (BMC) for the treatment of volumetric bone deficiencies in ten patients²⁵². They also found a rationale for a clinical application of BMC/bone aspirate in the treatment of osseous

defects. The intraoperative harvest procedure is a safe method and does not significantly prolong the time of surgery. *Dallari et al.* evaluated the use of bone grafts supplemented with platelet gel or platelet gel and bone marrow stromal cells for the healing of high tibial osteotomy. During the osteotomy, lyophilized bone chips with platelet gel were implanted into eleven patients (Group A), lyophilized bone chips with platelet gel and bone marrow stromal cells were implanted in twelve patients (Group B), and lyophilized bone chips without gel were placed in ten patients as controls (Group C). Six weeks after surgery, computed tomography-guided biopsies of the grafted areas were performed and the specimens were analysed by histomorphometry. Clinical and radiographic evaluation was performed at six weeks, twelve weeks, six months, and one year after surgery. The histomorphometry after six weeks showed significantly increased osteoblasts and osteoid areas in the cell group and the group with platelet gel compared to the control group, as well as increased bone apposition on the chips which was the highest in the cell group. Moreover, the cell group showed significantly higher revascularization than the controls. Radiographs revealed a significantly higher rate of osseointegration in the cell group and the group with platelet gel than in the control at six weeks. At the final evaluation at one year, the osseointegration was still better in the cell group and the group with platelet gel compared to the control group. However, all patients had complete clinical and functional evidence of healing.

In 2009, *Kim et al.* published their results of a randomized, multi-centre clinical study to compare the effect and safety of autologous cultured

osteoblast (Ossron™) injection to treat fractures. Sixty-four patients with long-bone fractures were randomly divided into two groups, i.e. those who received autologous cultured osteoblast injection and those who received no additionally cell-treatment. The cells were obtained during the fracture surgery via bone marrow aspiration (3-5ml) in all patients of the experimental group. The cells were cultured in an osteogenic differentiation media and after 8 weeks about 1.2×10^7 cells/0.4ml in combination with 0.4ml of fibrin were injected into the fracture area using a radiation imaging instrument (C-arm). The sum of the difference in the callus formation scores after four and eight weeks was used as the first efficacy variable. A modified callus formation score was used to evaluate the effectiveness of the injected cells. The average callus formation scores at the time of patient enrolment were 1.4 and 2.1, respectively, in the experimental and control group patients. The final average callus formation scores of the experimental and control groups were 7.1 and 5.8, respectively, which was statistically significant ($p = 0.03$). But at one month, the data were not statistically significant ($p = 0.196$). The conclusion of this study was that autologous cultured osteoblasts can be used as an effective method for accelerating the rate of fracture healing. But, the study lacks of the more interesting long term outcomes after 6 months and 12 months, and therefore the results should be construed carefully.

In the field of oral and maxillofacial surgery, *Warnke et al.* reported the successful transplantation of a customized, vascularised mandible replacement that had been exogenously engineered and subsequently cultured *in vivo* inside the patient's own 56-year-old body²⁵³. A titanium mesh

was chosen as an external scaffold, because degradable polylactide scaffolds had demonstrated a lack of contour stability in preceding animal studies. The custom-made titanium mesh was loaded with hydroxyapatite blocks that were coated with recombinant human Bone Morphogenetic Protein-7 (rhBMP-7) and bone marrow-derived mesenchymal stem cells. The patient's own body served as a living bioreactor as the prepared scaffold was implanted into his right latissimus dorsi muscle to allow for growth of heterotopic bone and for ingrowths of vessels from the thoracodorsal artery. After a cultivation period of 7 weeks the mandible replacement was transplanted (with the adjacent vessel pedicle) to repair a mandible defect resulting from resection of a tumour eight years previously. The patient regained full masticator function following this procedure allowing him to enjoy solids. Unfortunately, the patient died as a result of cardiac arrest 15 months after implantation of the mandible replacement.

All of the proposed techniques may be applicable for the repair of bone defects, and could be a useful alternative to autologous bone grafts. The methods described appear to be safe, minimally invasive, and easy to perform, with great potential in clinical applications. Unfortunately, due to practical and ethical reasons, clinical situations often lack proper controls, making data interpretation difficult. This aspect is still to be solved if clear-cut decisions have to be made while debating on technology transfer from the bench to the clinic.

Author	Year	No. of patients	Bone	Fixation	Biological enhancement	Size of Defect (average in cm)	Rate of union
Quarto et al. ²⁴⁶	2001	3	Tibia, Ulna, Humerus	EF	BMSC	5	100%
Vacanti et al. ²⁴⁸	2001	1	Phalanx	--	PC	2	100%
Hernigou et al. ²⁵¹	2005	60	Tibia	--	ABMG	--	88.3%
Morishita et al. ²⁵⁰	2006	3	Tibia Femur	no Fixation	MSC's	--	100%
Kitoh et al. ²⁵⁴	2007	11	Tibia, Femur	EF	CD+PRP+BMSC	8.95	100%
Dallari et al. ²⁵⁵	2007	12	Tibia	PO	BMSC	--	100%
Kim et al. ²⁵⁶	2009	31	Tibia, Femur, Humerus, Ulna, Radius, Fibula	IMN	Osteoblast (Ossron™)	--	100%

Table 4: Clinical application and outcomes of the treatment of bone defects and bone non-unions with intramedullary nailing (IMN), plate osteosynthesis (PO), callus distraction „Ilizarov technique“ (CD) and external fixation (EF) in combination with bone marrow derived mesenchymal stem cells (BMSC), platelet- rich plasma (PRP), autologous bone-marrow grafting (ABMG) and periosteal cells (PC).

2.5. Future Directions

Treatment of non-unions or segmental bone defects must consider above all the healing response of the tissue treated, in addition to the inductive properties of the graft. In fact, if the capacity to produce new bone is compromised, the induction phase will not be successful. Advanced age, infections, compromised soft tissue and all the conditions that cause a reduction in the number of cells for the regeneration process will require a combined approach. The use of autografts, autologous cells from bone marrow or, perhaps in the future, from mesenchymal cell banks will be an indispensable element that would enhance further non-union treatment strategies. The tissue engineering approach to bone defect repair, although successful and highly promising in most of the cases where it has been attempted, still needs rigorous evaluation and optimization. The results presented in the reviewed articles are not comparable. Rates of different

types of non-unions, time between initial injury and final intervention, differences in the size of bone defects and diversities on the application of each biologically enhancing modality make comparisons and conclusions difficult. Properly conducted clinical trials that include sufficient numbers of patients are warranted before claims regarding the therapeutic efficacy of mesenchymal stem cells and different types of growth factors for the treatment of large bone defects. As shown in several animal studies, bone tissue engineering has the potential to become a real clinical alternative to autologous bone grafts. Nevertheless, several problems have to be solved before translation into a routine clinical application. In large defects, the homogenous blood supply, including nutrients and oxygen, is still an unsolved problem. Additional *in vitro* studies, as well as further animal studies, should be performed to select required factors and different properties of the scaffold to achieve the best and constant supply of cells with nutrients. Regarding the tissue engineering aspect of bone regeneration, the size of the defect plays another important role. Fractures and non-unions are smaller than large segmental bone defects, and other treatment strategies are required. It might be sufficient, to use growth factors like BMP-2 or BMP-7 as local bone supplements to gain full bone regeneration.

In large bone defects, scaffolds are necessary for the replacement of the structural integrity. These scaffolds must provide sufficient initial mechanical strength and stiffness to substitute for the loss of mechanical function of the diseased or damaged tissue. Yet, scaffolds may not necessarily be required to provide complete mechanical equivalence to healthy tissue, but stiffness

and strength should be sufficient to at least support and transmit forces to the host tissue site in this context. Continuous cell and tissue remodelling is important for achieving stable biomechanical conditions and vascularisation at the host site. Hence, the 3D scaffold/tissue construct should maintain sufficient structural integrity during the *in vitro* and/ or *in vivo* growth and remodelling process. The degree of remodelling depends on the tissue itself (e.g. skin 4-6 weeks, bone 4-6 months), and its host anatomy and physiology. In addition to these essentials of mechanics and geometry, a suitable construct will possess a 3D and highly porous interconnected pore network with surface properties which are optimized for the attachment, migration, proliferation and differentiation of cell types of interest and enable flow transport of nutrients and metabolic waste. Furthermore, they should be biocompatible and biodegradable with a controllable rate to compliment cell/tissue growth¹³².

It cannot be emphasised strongly enough how essential it is to control and understand this degradation process to lead to successful tissue formation, remodelling, and maturation at the defect site. In the early days of tissue engineering it was believed that the scaffold should degrade and vanish as the tissue is growing. Currently, tissue ingrowth and maturation differs temporally from tissue to tissue and secondly tissue ingrowth does not equate to tissue maturation and remodelling, in other words a defect filled with immature tissue should not be considered “regenerated”. Hence, many scaffold-based strategies have failed in the past as the scaffold degradation was more rapid than the tissue remodelling and/or maturation¹³².

Mesenchymal stem cells are becoming more and more popular as a cell source for a cell-based tissue engineering approach. These cells have shown their therapeutic capacity in several *in vitro* as well as *in vivo* studies for the regeneration of bone defects and non-unions. The supply of autologous mesenchymal stem cells is often limited. But their special immunological characteristics suggest that MSC's could be used in non-autologous applications^{257,258}. This offers a new opportunity for the availability of mesenchymal stem cells for regenerative medicine and a clinical application.

Over the last few years, some research groups have focused their work on some additional cell sources. Osteoblasts isolated from axial bones and from orofacial bones show in some *in vitro* studies a higher osteogenic potential than mesenchymal stem cells. Further experiments are required regarding cell sources in combination with different scaffolds and growth factors. With a more established foundation of knowledge, researchers will be able to answer questions about whether cells for implantation should be undifferentiated or differentiated, and what the optimal cell source, proportion, and method of implantation will be to translate research findings into clinical practice. For the clinical application of different novel treatment strategies including the tissue engineering approach, a good clinical outcome with recovery of nearly normal function of the limb is most important for the patient. Prior to treatment of a patient with a bone defect or bone non-union, the different available options for treatment, even non-operative treatment, have to be balanced, and the best option should be taken in regard to the

patient's situation. Therefore, a close cooperation between researchers and clinicians is necessary to use the optimal combination of different treatment strategies that are available for the patient.

Chapter III - Benchmarking autologous vs. allogenic mesenchymal progenitor cells for the reconstruction of critical-sized segmental tibial bone defects in aged sheep

3.1. Introduction

There are two major engineering approaches in the development of novel treatment concepts using scaffolds; cell-based and cell-free. The most suitable cell source for scaffold-based bone tissue engineering is still focus of much debate in the literature. The nature of regenerative medicine will be profoundly influenced by whether the cells used are allogeneic or patient-autologous. It is unlikely to be wholly one or the other that will dominate, however, at this early stage it is worth examining the issues, both from a scientific and translational point of view.

A great advantage of natural patient-derived (autologous) cells is twofold; firstly the cells can be readily prepared either in the operation theatre or in a special lab attached to the hospital. Hence autologous therapy is something that clinician scientist can proceed in house, at least to early clinical trials, although they still need to meet regulatory requirements and secondly no host-versus-graft immunological reaction⁷⁹.

For universal (allogeneic) cell material the pronounced advantage is that it is qualified of representing a commercial technology more comparable with that of pharmaceutical drug than autologous cells. This is because it is possible to envisage manufactured goods by scale-up with product characterization, which defines safety, applied to sizeable batches of cell-based products. In addition, in medical emergencies allogeneic cells have quite a lot of supplementary advantages. First, given reasonable cell stability during storage, there will be an immediately available supply in the operation theatre. A further value in non-elective treatments is that these therapies do not require a preliminary biopsy from a potentially severely ill person, thus avoiding an additional procedure and all the associated challenges including appropriate patient biopsy consent⁷⁹.

In bone tissue engineering cell-based strategies aim to improve osteoinduction by the incorporation of cells with a high osteogenic differentiation potential such as bone marrow derived mesenchymal progenitor cells (bMPCs). Gronthos et al.²⁵⁹ have defined these cells as multipotent progenitor cells, which have the potential to differentiate into a variety of mesenchymal tissues such as bone, cartilage, tendon, ligaments, muscle, fat and dermis^{80,81,82,83}. They can be isolated from a variety of tissues^{233,234,235} using different separation techniques and can be differentiated into the appropriate phenotype under defined culture conditions and the action of specific growth factors or cytokines²³⁶. These cells have

shown their therapeutic potential in a number of *in vivo* studies for the regeneration of large bone defects and non-unions^{239,242,260-262}.

The supply of autologous MPCs is often limited and the preoperative preparations for their isolation, expansion and differentiation is time consuming and expensive. Hence, to acquire an adequate amount of cells for transplantation, the time period between cell isolation and cell transplantation is usually at least 4 – 6 weeks. Therefore, the major drawback of using an autologous cell source is two-pronged; limitations in cell numbers when utilising them immediately after extraction or the long time period and associated costs, which are necessary to expand the cells *in vitro* until a suitably high number is attained. However, the special immunological characteristics, which are evident with MPCs, suggest that MPCs could in fact be used successfully for non-autologous applications^{257,258}. Allogenic cell transplantation is a common therapeutic option and is in routine clinical use in the field of oncology^{263,264}. Translating the idea of allogenic cell-transplantation from oncology to orthopaedics could offer a new opportunity for the availability of MPCs for regenerative medicine as an “off the shelf product”. Before translating these new treatment concepts into a clinical application in orthopaedic and trauma surgery, rigorous evaluation of the respective cell populations in an adequate preclinical animal model are essential^{76,77}.

Several animal models have been developed over the years to verify the practicality of different research approaches in bone regeneration. Among these, adult sheep offer the advantage of having a comparable body weight, a similar mineral composition of bone and similar metabolic and remodelling

rates to humans and furthermore, long bone dimensions suitable for the use of human implants and prostheses, which is not possible in smaller species^{65,265}. Thus, our group has recently established a challenging ovine segmental bone defect model using relatively old animals, which show the secondary osteon remodelling characteristic of human bone. We move towards defining an appropriate cell source for bone tissue engineering to circumvent the aforementioned disadvantages associated with autologous cell transplantation in favour of allogenic MPC sources²⁶⁶.

We hypothesize that allogenic MPCs have a similar osteogenic potential compared to autologous MPCs and can be used in a scaffold-cell based bone tissue engineering concept. Therefore, the aim of the current study was to assess and compare the regenerative potential of autologous versus allogenic MPCs in combination with a mPCL-TCP scaffold in a critical sized segmental bone defect in a sheep tibia.

3.2. Material and Methods

3.2.1. Scaffold fabrication and preparation

Bioresorbable cylindrical scaffolds of medical grade poly-caprolactone (80%) and β -tricalcium phosphate (20%), (mPCL–TCP), (outer diameter: 20 mm, height: 30 mm, inner diameter: 8 mm) are used in this study. The scaffolds are produced by fused deposition modelling (FDM) and obtained from Osteopore International (Osteopore International, Singapore). The scaffolds have a porosity of 70% and a 0/90° lay down pattern (Fig. 3). This architectural layout is particularly suitable for load bearing tissue engineering

applications since the fully interconnected network of scaffold fibres can withstand early physiological and mechanical stress in a manner similar to cancellous bone. Moreover, the architectural pattern allows retaining of coagulating blood during the early phase of healing, and bone ingrowth at later stages. Prior to surgery, all scaffolds are surface treated for six hours with 1M NaOH and washed five times with PBS to render the scaffold more hydrophilic. Scaffold sterilization is achieved by incubation in 70% ethanol for 5 min and UV irradiation for 30 min.

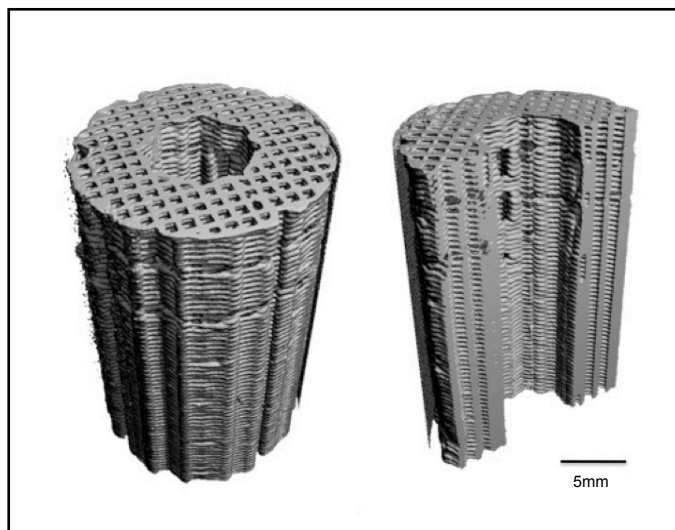


Figure 3: MicroCT image of a cylindric mPCL-TCP scaffold produced via fused deposition modelling for segmental bone defect repair.

3.2.2. Animal study

Thirty-two male sheep (40-50 kilogram, age 7-8 years) were used in this project (Table 5). An Animal Ethics Approval Certificate has been obtained. Animal surgery was performed at the QUT Medical Engineering Research Facility, The Prince Charles Hospital, Chermside. Owing to ethical reasons to lower the number of animals, the results of the experimental groups will

additionally be compared to the scaffold only group (negative control group) and the group with autologous bone graft (ABG), (positive control group) from the work of Dr. Johannes Reichert, who did these experiments for his thesis. There will be 8 animals in each group and all will be euthanased after 3 months.

The research plan for this study is structured as follows:	Group I	Group II	Group III	Group IV
	mPCL-TCP	mPCL-TCP autologous MPC	mPCL-TCP allogenic MPC	ABG
Total	8	8	8	8

Table 5: The research plan with details of the animal treatment groups.

3.2.3. Preparation of platelet rich plasma

To produce platelet rich plasma (PRP) 80ml of blood was collected from the jugular vein of each sheep and transferred into 3.5-ml monovettes supplemented with sodium citrate (3.8%) at a ratio of 9 volumes blood and 1 volume sodium citrate according to Anitua et al.⁸⁸ (Fig. 4 A). The citrated blood of 30 monovettes was transferred to eight 15 ml falcon tubes and centrifuged in a standard laboratory centrifuge for 20 min at 2,400 rpm (Fig. 4 B/C). Subsequently, the yellow plasma of all tubes was transferred to one new 50-ml falcon tube and platelets pelleted in a second centrifugation step for 10 min at 3,600 rpm (Fig. 4 D)²⁶⁷. The pellet was resuspended in 2ml of plasma and the platelets counted in a hemocytometer. After the preparation,

PRP was mixed with the cells (MPC), and the resulting suspension was loaded onto the sterilized scaffolds (Fig. 4 E/F). PRP was activated using Thrombin (5U/ml), and the scaffolds placed in the incubator for at least one hour before the operation.

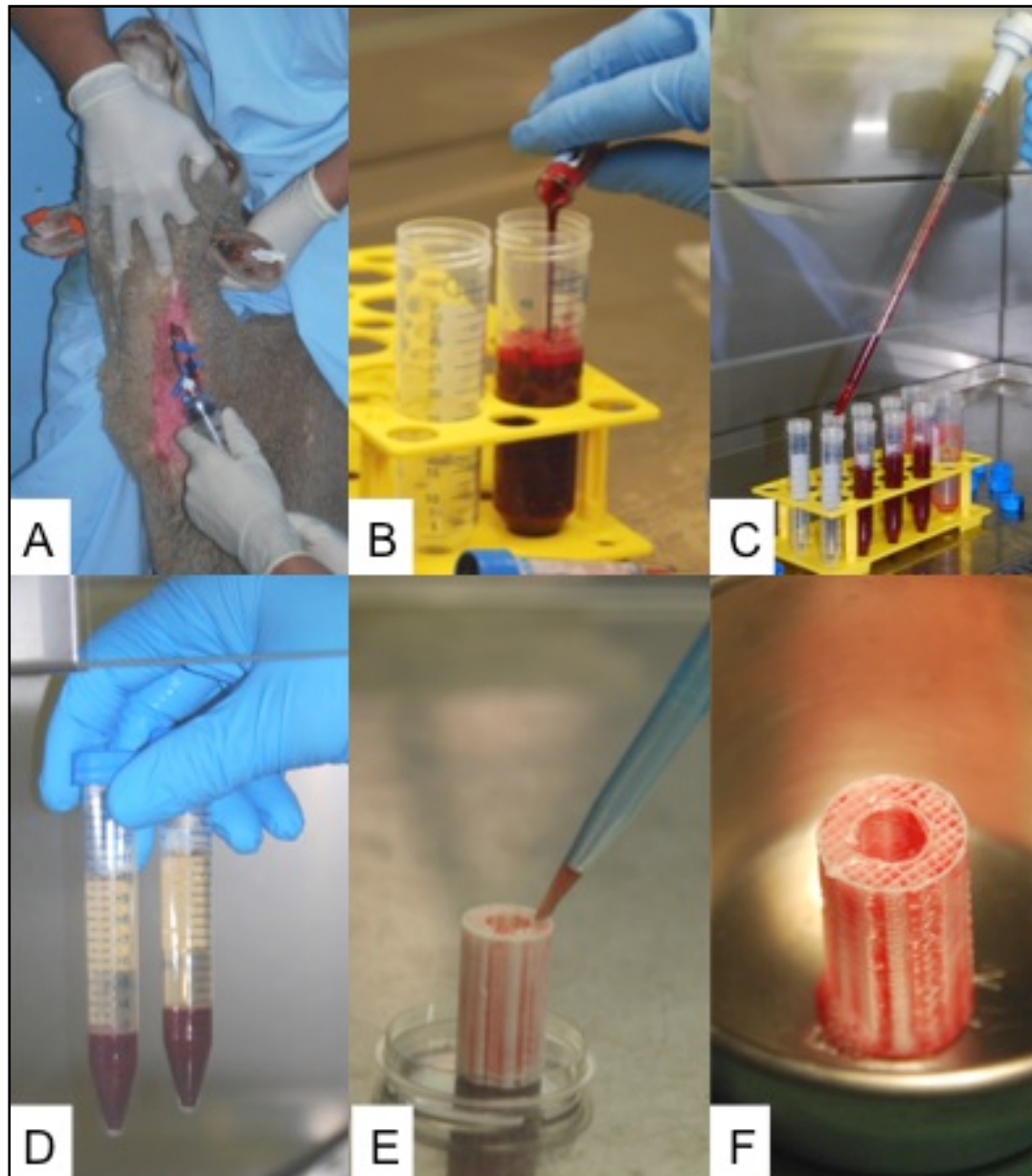


Figure 4: Blood is collected from the jugular vein of the sheep (A), mixed (B), and transferred into eight 15 ml falcon tubes (C). After centrifugation with 2400 rpm for 20min, the plasma is removed and centrifuged for a second time (D). The resulting pellet is resuspended in 2ml of plasma, and the cells in combination with PRP seeded onto the scaffolds (E/F).

3.2.4. Animal Surgery

3.2.4.1. Anaesthesia and pre-operative treatment

A jugular venous line is installed under aseptic conditions, and 15-20 ml of 1% propofol injected to induce general anaesthesia. The animal is intubated with a 9-10 mm silicon endotracheal tube and connected to an automatic respirator (Campbell anaesthetic ventilator) for assisted ventilation with 2l O₂/min. The general anesthesia is maintained with propofol (2%) at a rate of 120-140 ml/min. For analgesia, Buprenorphine (0.1 mg per 10 kg body weight) is administered, for antibiotic prophylaxis gentamycine (5 mg/kg) and cephalothin (25 mg/kg). The animal's ECG, heart rate, oxygen saturation and end-tidal carbon dioxide levels are monitored and recorded continuously.

3.2.4.2. Surgical Procedure

All animals are placed in the right lateral position. The right hind limb is carefully shaved and thoroughly disinfected with 0.5% chlorhexidine solution red in 70 % ethanol. The animal torso and surroundings are covered with sterile sheets, the surgical area additionally with Opsite (Smith and Nephew). The right tibia is exposed by a longitudinal incision of approximately 12 cm length on the medial aspect of the limb (Fig. 5 A). A bone fixation plate (4.5 mm broad DCP, 10 holes, Synthes) is adjusted to the morphology of the bone by bending (plate-bending press, Synthes) and applied to the medial tibia. The distal end of all plates is placed 2.5 cm proximal of the medial malleolus (Fig. 5 B). The screw holes are drilled and the plate is temporarily fixed with 2 screws adjacent to the anticipated defect (Fig. 5 C). The middle of the defect site is measured and marked with a raspator. A distance of 1.5

cm each proximally and distally of the defect middle is measured and marked to define the osteotomy lines (Fig. 5 D). Next, the soft tissue inserting to the bone in the designated defect area is detached and a wet compress placed between bone and posterolateral soft tissue to avoid damage to proximate nerve and blood vessels during osteotomy (Fig. 5 E). Parallel osteotomies perpendicular to the bone's longitudinal axis are performed with an oscillating saw (Stryker) under constant irrigation with saline solution to prevent heat induced osteonecrosis whilst the bone segment of 3 cm length is excised (Fig. 5 F). Care is taken to completely remove the periosteum within the defect area and 1 cm proximally and distally of the osteotomy lines (Fig. 5 G/H). The plate (DCP) is fixed on the proximal bone using 4 screws. Afterwards, the scaffold is gently placed into the defect and the distal part of the tibia is fixed to the plate using 3 screws (Fig. 5 I/K). Care is taken not to destroy or damage the scaffold but to place it, press fit, between the proximal and distal part of the tibia. The wound is closed in layers with a 2-0 Monocryl (Ethicon) and a 3-0 Novafil (Syneture) suture for the skin. The closed wound is sprayed with Opsite (Smith and Nephew), covered with pads and bandaged (Vetrap, 3M). After recovery from anaesthesia, animals are allowed unrestricted weight bearing.

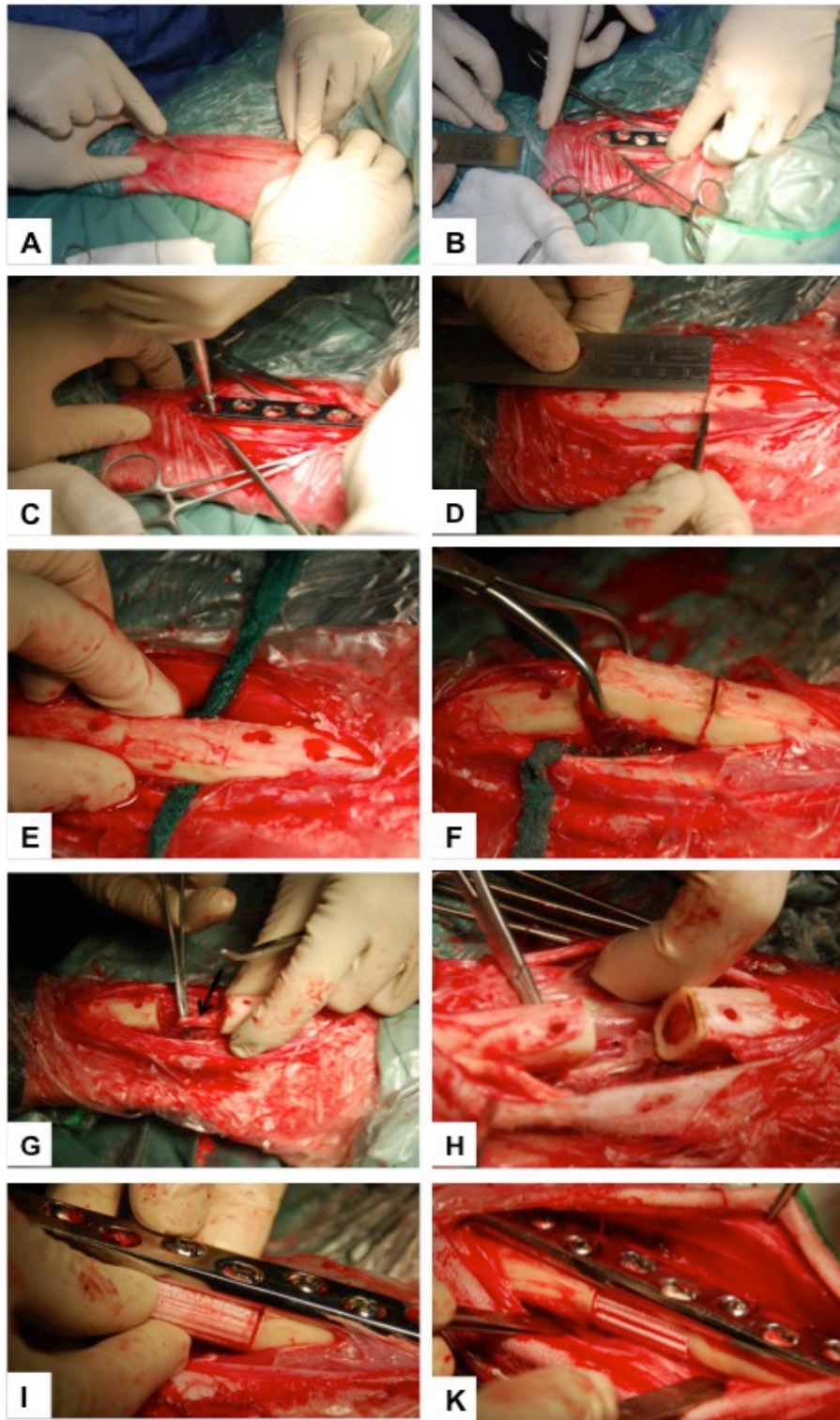


Figure 5: To create a 3 cm segmental defect, a skin incision is made over the medial part of the tibia (A), the plate is placed 2.5 cm proximal of the malleolus mediales (B) and temporarily fixed with two screws (C). The screw holes are drilled and the defect middle and osteotomy lines are marked (D). A wet compress is placed between the bone and the posterolateral soft tissue, to avoid damage to the nerve and blood vessels (E). The bone segment is removed after osteotomy (F). Care is taken to remove the periosteum (black arrow) (G), which is in the dorsal part very closed to the vessels and the nerve (black star) (H). After implanting the scaffold (I), the bone fragments are realigned and fixed with a plate and screws (K).

3.2.4.3. Euthanasia

The animals were euthanized after 3 months by intravenous injection of 60 mg/kg pentobarbital sodium (Lethobarb, Virbac, Australia). After euthanasia, both hind limbs were exarticulated at the knee-joint, the tibia dissected and the fixation plate removed.

3.2.5. Experimental Groups

- Group I

The empty scaffold (mPCL-TCP) was placed into the defect and the wound closed in layers.

- Group II

Ovine mesenchymal progenitor cells (MPC) were obtained from Merino sheep undergoing experimental surgery. Bone marrow aspirates were obtained from the iliac crest under general anaesthesia (Fig. 6). Total bone marrow cells ($5-15 \times 10^6$ cells/ml) were plated at a density of $10-20 \times 10^6$ cells/cm² in complete medium consisting of low glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were subsequently plated at a density of 10^3 cells/cm². Two weeks before implantation, the medium was changed to an osteogenic media (DMEM, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 10 µl/ml β-glycerophosphate, 1 µl/ml ascorbic acid and 1 µl/ml dexamethasone) to induce osteogenic differentiation. For 3D cultures, 35×10^6 ovine MPC suspended in 250 µl of basal medium were mixed with PRP and then seeded onto each collagen type I coated mPCL-TCP scaffolds. PRP was activated

with thrombin and the cell scaffold constructs were implanted into the defect site and the wound closed in layers around the autograft.



Figure 6: Bone marrow aspiration from the iliac crest under general anaesthesia.

- Group III

The cells (allogenic MPC) used in this group were obtained from merino sheep that were not included in this group (allogenic cells). The procedure of cell harvesting, cell culture and scaffold preparation is the same as explained in group II.

- Group IV

Autologous cancellous bone graft (ABG) was harvested from the left iliac crest. The surgical area was shaved and disinfected with 0.5% chlorhexidine red in 70% ethanol. A 5 cm incision was made following the iliac crest, the inserting musculature was carefully detached and the cortical bone of the lateral *os ileum* was fenestrated (2 x 2 cm) using a hammer and osteotome. Care was taken not to fracture the *ala ossis ilii*. The resulting lid was carefully

removed with a raspator and the cancellous bone harvested utilizing a bone curette. Then, the lid was reinserted, the musculature reattached with 2-0 Vicryl sutures (Ethicon), and the wound closed in layers. The closed wound was sprayed with Opsite (Smith and Nephew).

3.2.6. Blood analysis

The allogenic group was monitored for six weeks after the procedure to assess any reaction to the impantation of the cells. Blood samples were taken from all animals of the allogenic group and of the autologous group as a control. Blood samples were taken on day 1,3,7,14,21 after the operation. At the same time the sheep were physically examined. A final physical examination was performed 6 weeks after surgery.

3.2.7. Radiography analysis

Throughout the study, x-rays were taken after 6 and 12 weeks, to determine the time of bridging of the defect in the different experimental groups. Conventional x-ray analysis (3.2 mAs; 65kV) was performed in two standard planes (anterior-posterior and medial-lateral). At euthanasia, the gross morphology and mobility at host-graft junctions was clinically assessed and the findings carefully documented and photographed.

3.2.8. Computed tomography (clinicalCT)

After sacrifice, a clinical CT scanner (Philips Brilliant CT 64 channels) was used to scan the experimental limbs. A dipotassium phosphate phantom was

used to calibrate measurements of mineral density. 3D reconstructions from the CT data were generated with AMIRA® 5.2.2 (Visage Imaging GmbH, Berlin, Germany) (Visage Imaging) with a threshold of 300 and qualitative analysis was performed to assess mineralization within the defect and bridging. For quantitative analysis, the CT datasets of operated and contralateral intact tibia of each animal were first cropped to image stacks with equal bounding box dimensions using AMIRA®. Next, cortical bone and callus tissue were segmented by choosing appropriate threshold values (lower threshold: 300) for the measured grey levels. A 3D surface was generated and saved as a binary file (STL binary Little Endian format). These .stl files were loaded into Rapidform2006 (Inus Technology, Seoul, Korea) and 4 corresponding reference points were selected on each intact and defect tibia and bound to the respective shell. Intact and defect tibia were registered to align their shells utilizing the previously defined common geometries between them. The reference point coordinates of the defect tibia were recorded prior to and after registration. The coordinates of the initial and final points were entered in an in-house MATLAB program (MATLAB 7.6.0, MathWorks, Inc., Natick, MA, USA) to determine the matrix for the required transformation. This transformation matrix was then used to align the image data stacks in AMIRA®. This alignment results in the intact and defect tibia having the same orientation and allows the definition of the three different regions of interest within the defect. Next, the amount of newly formed bone in these three different regions of interest within the 3 cm defect area was calculated using an in-house MATLAB program. Region 1 is the region of the

cortical bone, region 2 is the bone marrow region, and region 3 is the external bone formation around the defect (Fig. 7).

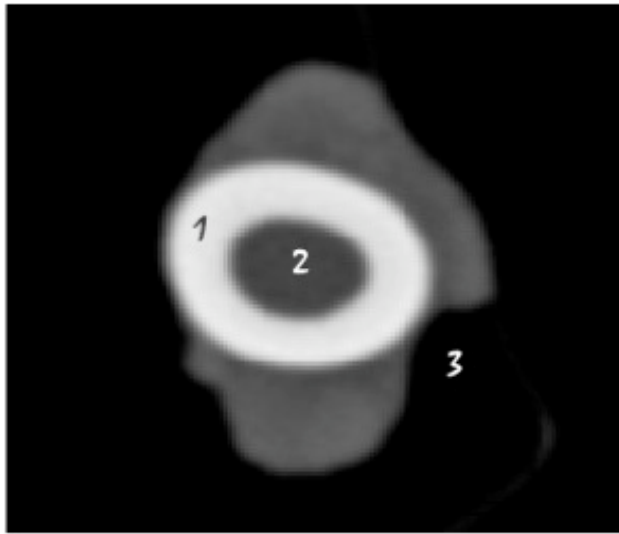


Figure 7: CT DICOM image of an intact ovine tibia (axial view) defining the three regions of interest. Region 1 is the region of the cortical bone, region 2 is the bone marrow region, and region 3 is the external bone formation around the defect

3.2.9. Biomechanical evaluation

To determine the integration of the scaffolds into the bone and the recovery of the biomechanical function of the affected tibias, biomechanical testing was performed. After euthanasia, both tibias of each sheep were explanted and the fixation plate of the experimental leg was carefully removed. Both ends of the tibiae were embedded in 80 ml Paladur (Heraeus-Kulzer GmbH) and each bone was then mounted in an Instron 8874 biaxial testing machine (Fig. 8). By leaving as much soft tissue as possible attached, bone samples were prevented from drying out. Next, for the experimental and the intact tibia (control) a torsional test until failure was performed in a biaxial universal testing machine (Instron 8874, Instron, Norwood, USA). The torsion test was conducted with a compressive load of 0.05kN and at an angular velocity of

0.5 deg/s until the fracture point was reached (right tibiae counter clockwise, left tibiae clockwise). In order to avoid the possibility of damage to the specimens in the early stages of bone healing, no preconditioning of the samples was performed. The torsional moment (TM) and torsional stiffness (TS) values were calculated from the slope of the torque-angular displacement curves and normalized against the values of the intact contralateral tibiae.

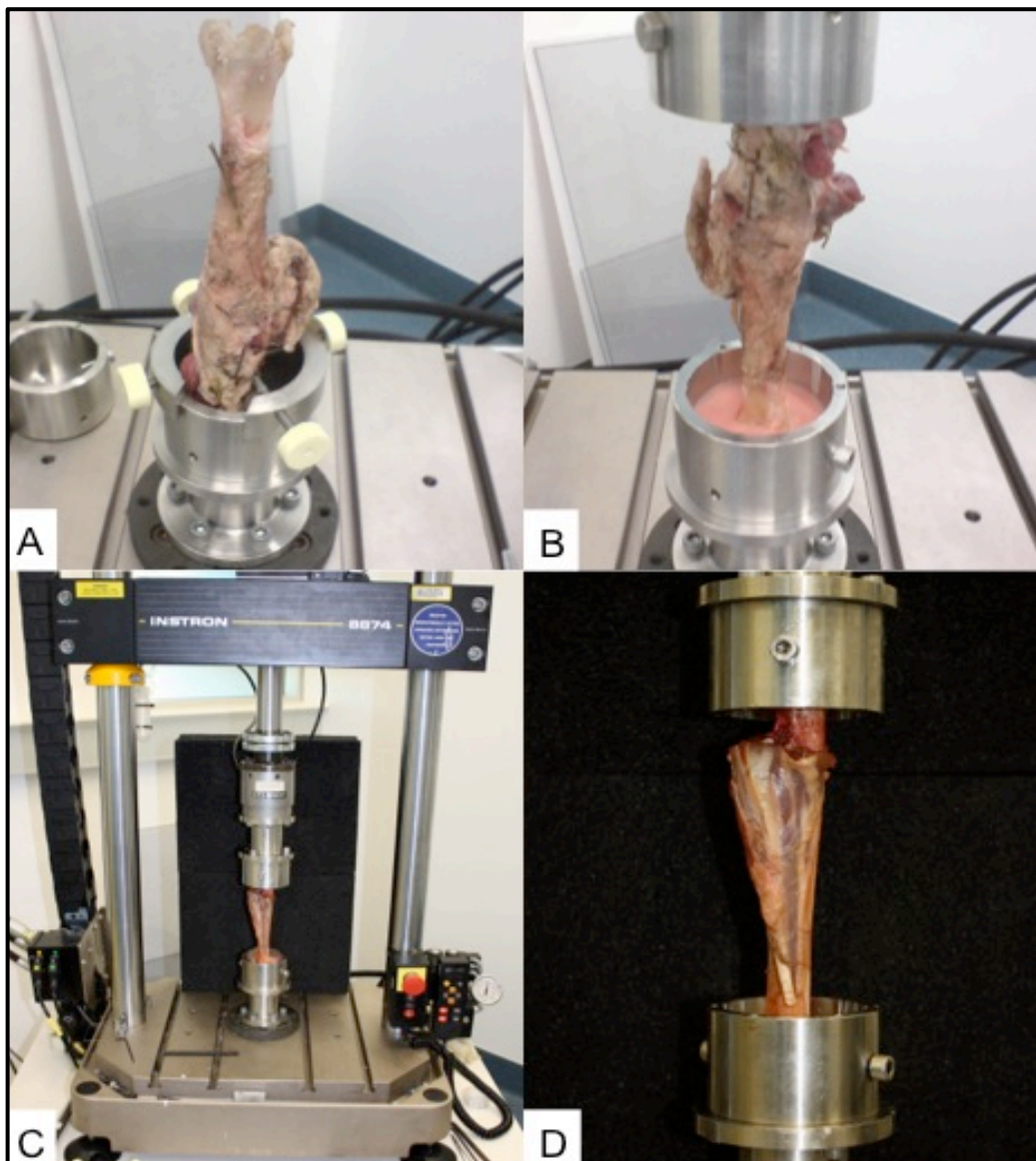


Figure 8: Potting of a sample tibia for biomechanical testing. First, the proximal part is embedded in Paladur with the tibial axis vertically aligned (A). The tibia is then rotated and the distal part is embedded (B). The torsion test is conducted counter clockwise for the right tibiae and clockwise for the left tibiae (C-D).

3.2.10. Computed tomography (microCT)

After mechanical testing, both tibias were fixed in 10% neutral buffered formalin (NBF) and micro-CT scans of the defect and the control side were performed. On the defect side, a region of interest including the 30 mm defect gap and 5 mm of adjacent bone each proximally and distally was selected. A 1 cm large piece of bone from the contralateral side was scanned as a control. Samples were scanned (vivaCT 40, Scanco medical) with a voxel size of 36 μm . Samples were evaluated at a threshold of 210, a filter width of 0.8 and filter support of 1.0 and analysed for bone volume and bone mineral density within the defect using the software supplied by the manufacturer of the μCT . For bone volume, the regions of interest were determined as external callus formation (external), bone formation within the scaffold (scaffold) and bone formation in the inner part of the scaffold (endosteal) (Fig. 9).

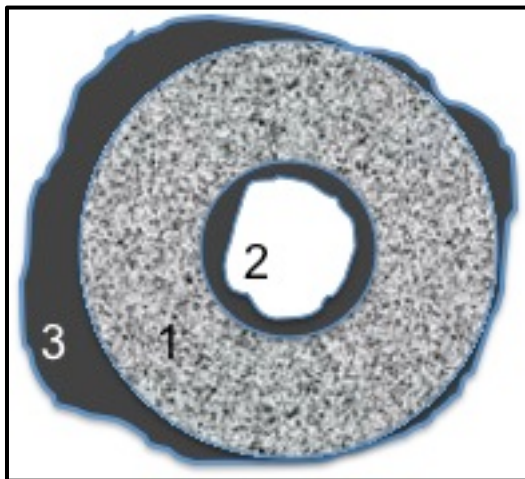


Figure 9: For bone volume evaluation, three regions of interest were determined as external callus formation (external, 3), bone formation within the scaffold (scaffold, 1) and bone formation in the inner part of the scaffold (endosteal, 2).

3.2.11. Histological analysis

After biomechanical testing and microCT analyses, tibial bone specimens were trimmed to 8 cm length. For histological analysis, the mid-defect regions were sectioned in the transverse and sagittal plane. Half of the samples were used for paraffin sectioning. After decalcification in 15% EDTA for 6-8 weeks, the samples were embedded in paraffin and 5 µm cross sections used for histological/histomorphometry (H&E and Masson's Trichrome stains respectively) and immunohistological analysis. The other half of the samples were used for undecalcified embedding in methylmethacrylate. Longitudinal and cross sections were performed with a thickness of 6 µm and stained with Safranin Orange/von Kossa and Movat's pentachrome to identify new bone formation.

3.2.12. Statistical analysis

Statistical analyses for the biomechanical results and for the ct-scans (clinicalCT and microCT) were carried out using a two-tailed Mann-Whitney-U-test (SPSS 16.0, SPSS Inc.) and p-values are adjusted according to Bonferroni-Holm. Results were considered significant for p-values <0.05.

3.3. Results

3.3.1. Cell Isolation and Differentiation

Mesenchymal progenitor cells were obtained from the sheep undergoing experimental surgery via bone marrow aspiration from the iliac crest under general anaesthesia (Fig. 10 A). Total bone marrow cells were plated at a density of $1-2 \times 10^7$ cells/cm² in complete medium and cultured until they were confluent (Fig. 10 C). Two weeks before implantation, the medium was changed to an osteogenic media to induce osteogenic differentiation. Within a few days, the cells showed a clear response to the osteogenic induction media by a pronounced morphological change. The cell morphology changed from an elongated shape to a compact cobblestone-like appearance (Fig. 11). The potential of bone marrow derived MPCs to secrete a mineralised extracellular matrix was analysed by alizarin red staining. After 2 weeks of induction, cultured MPCs had formed extensive amounts of alizarin red-positive mineral deposits throughout the adherent layers (Fig. 12).

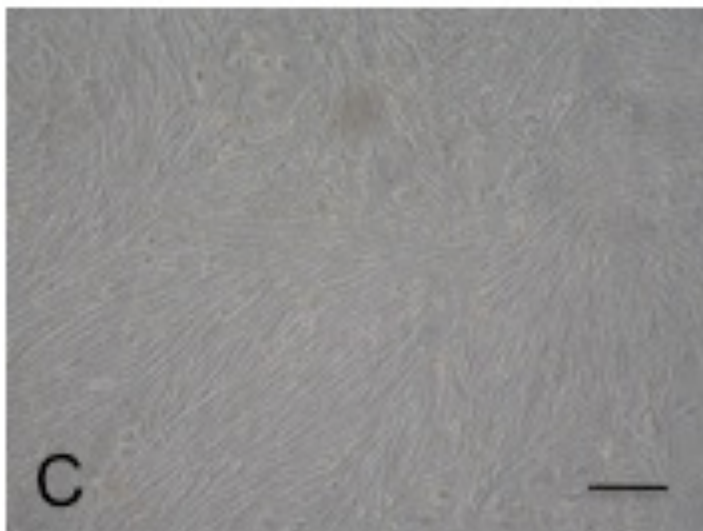
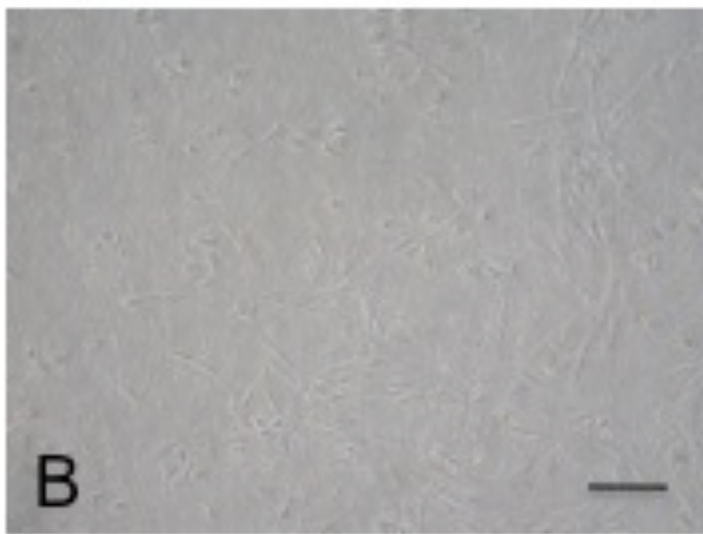
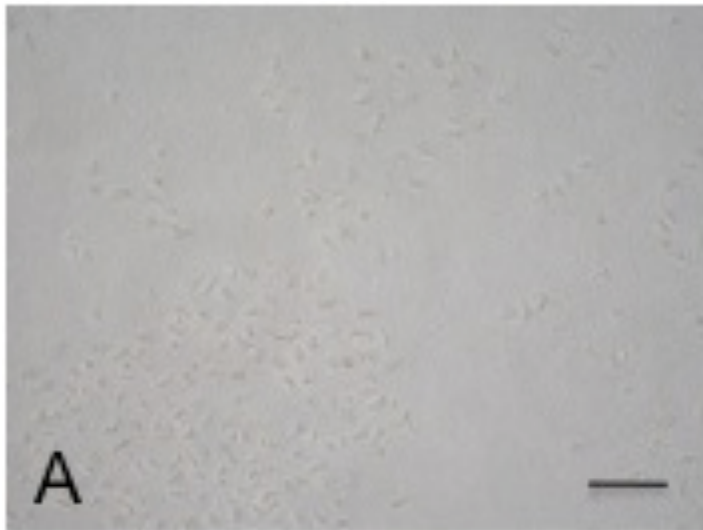


Figure 10: Light microscopy images of the culture of mesenchymal stem cells (A) 3 days, (B) 7 days, and (C) after 14 days, (scale bar = 100 μm).

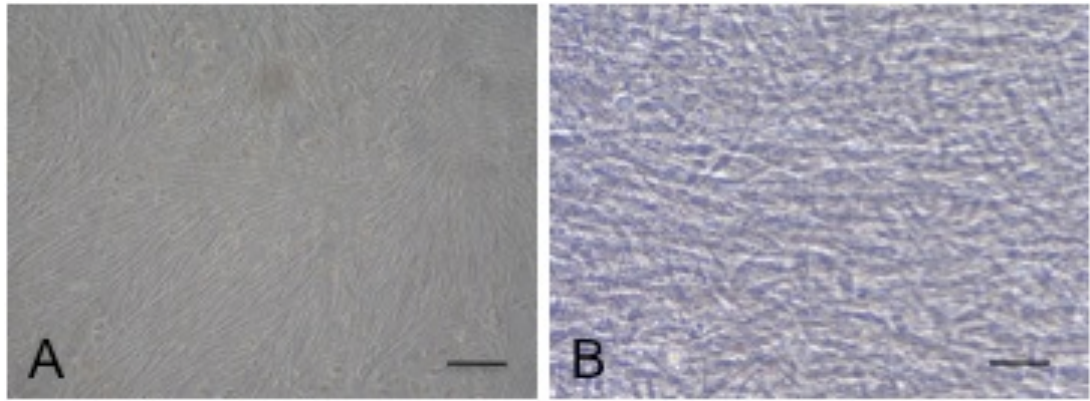


Figure 11: The cells typically have an elongated shape in regular media (A), this changed to a compact cobblestone-like appearance within days of being exposed to the osteogenic media (B), (scale bar = 100 μm).

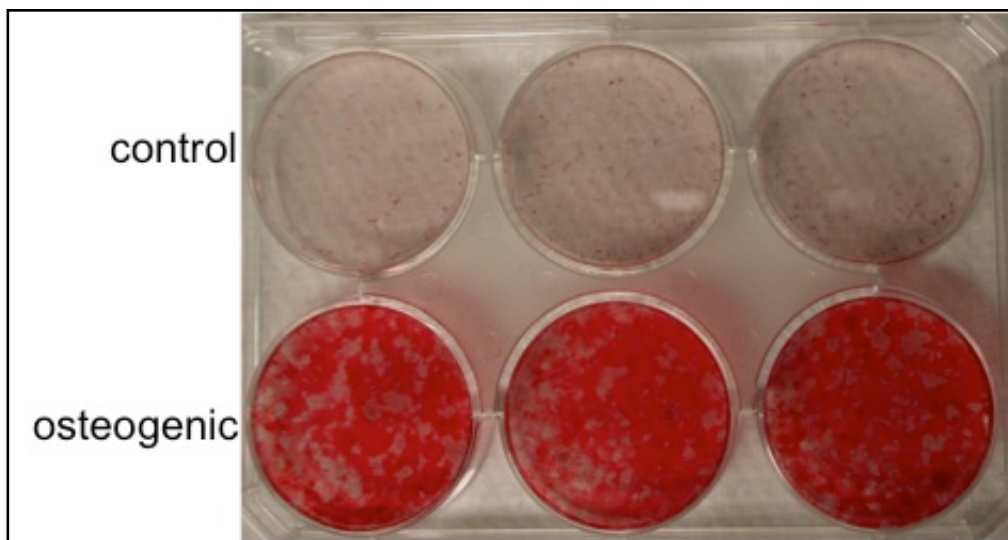


Figure 12: Alizarin red staining of mineral deposits for MPC cultures after 14 days on 6-well plates. Under osteogenic conditions (osteogenic), MPC's secrete an alizarin-red stained mineralized matrix. The control cultures (control) stained negative.

3.3.2. Animal Surgery

In all animals, no postoperative infections or other complications were observed. The chosen 4.5 mm broad DCP was proven to be biomechanically sufficient to prevent implant failure. All animals were in good health and survived the experimental period gaining weight in the months following

surgery. In particular, the animals of the allogenic group showed no clinical signs of implant rejection.

3.3.3. Blood analyses

Venous blood samples were taken preoperative and on day 1, 3, 7, 14, 21 after the operation from all the animals of the allogenic group and some animals of the autologous group as a control value. The blood analyses showed no signs of graft rejection. Preoperatively, the result of the white cell count (WCC) in the allogenic group were slightly higher compared to the autologous group, and did not increase more than the autologous group after surgery (Fig. 13).

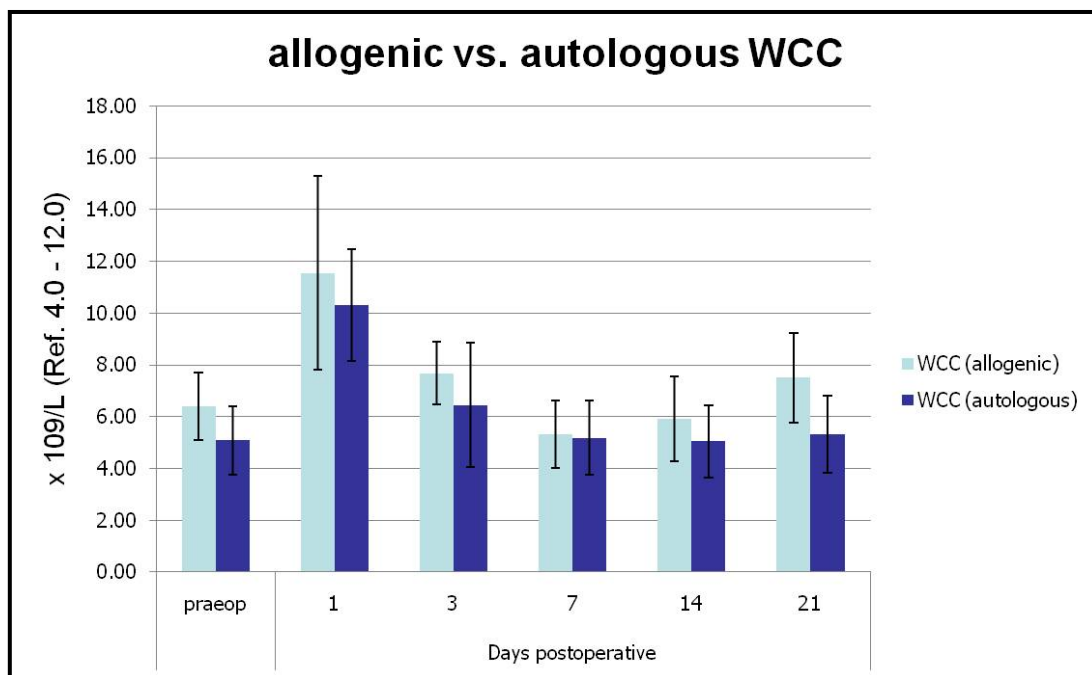


Figure 13: White cell count (WCC) of venous blood samples of animals of the autologous (dark blue) and allogenic (light blue) experimental groups. No significant differences were found between the groups.

3.3.4. Radiographic analysis

Immediately after surgery, after 6 and 12 weeks, conventional x-ray analyses in two standard planes (anterior-posterior and medial-lateral) were performed

to assess bone formation. After surgery, the correct position of the scaffold, the plate and the screws were confirmed. The x-ray analysis after 12 weeks showed no loosening of the implants or movement of the scaffolds in all experimental groups. External callus and bone formation within the defect was observed in all animals of the autologous and the allogenic cell group (Fig. 14 B/C). But a complete bridging of the defect was only observed in one animal of the allogenic group and two animals of the autologous group. The groups treated with mPCL-TCP scaffold (A) showed no radiographic signs of bone formation within the defect. The defect treated with autologous bone graft (ABG) showed clear radiographic signs of bone formation with defect bridging in the x-rays (E).

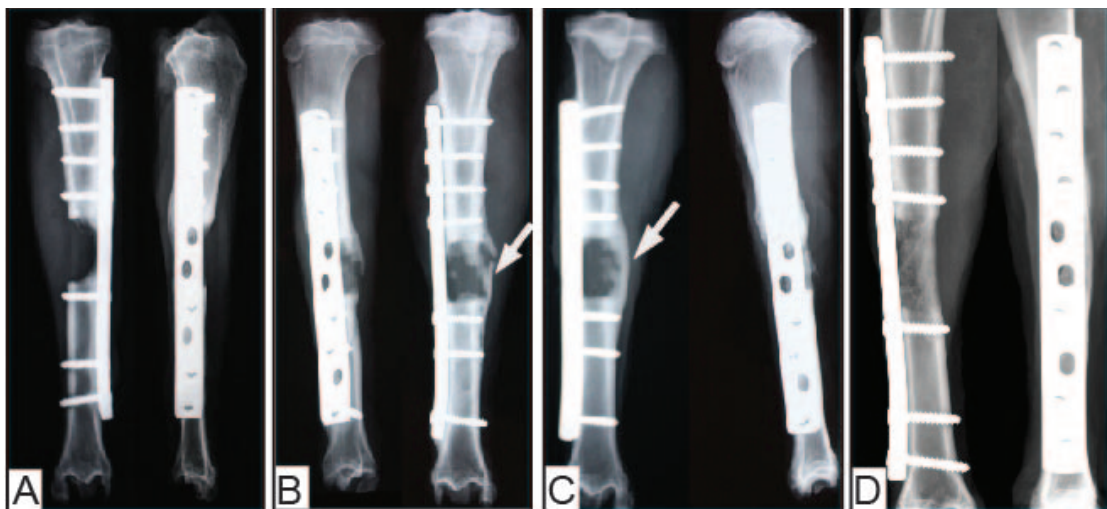


Figure 14: Representative x-ray images of the experimental groups after 3 months. The groups treated with the scaffold only (A) showed no bone formation within the defect after 3 months. The defects reconstructed with mPCL-TCP scaffolds seeded with autologous MPC's (B) and with allogenic MPC's (C) show clear radiographic signs of a beginning defect bridging in both cell groups (white arrow) on the site of the vascular bundle (enhanced vascularity). The defect treated with autologous bone graft (ABG) showed the highest amount of bone formation in the defect with clear radiographic signs of defect bridging (D).

3.3.5. Computed tomography (clinicalCT)

Qualitative CT analysis after 12 weeks showed bone formation within the defect in both experimental groups (Fig. 15). In two animals of the autologous cell group and in one animal of the allogenic cell group a defect bridging had occurred, which was on the posterolateral side. The results were additionally compared with the experimental groups of previous experiments of our group. Minor bone formation was seen in the mPCL-TPC scaffold group (negative control group), whereas a full defect bridging had occurred in all defects reconstructed with ABG (positive control group). No radiographic signs of inflammation (e.g. diffusely delimited soft tissue infiltrations, osteolysis, osteomyelitis, abscesses) were found. Scaffolds showed good osseointegration without any signs of resorption.

Median values of total bone volume (BV) in the defect were higher in the allogenic cell group compared to the autologous cell group (Fig. 16). Furthermore, both cell groups showed a higher total bone volume compared to the scaffold only group. All the differences were not significant. In the cortical region (region 1) both cell groups showed a higher bone formation compared to the scaffold only group. Bone formation in the ABG group was calculated to be significantly higher when compared to the scaffold only group. Bone formation in region 2, the marrow region, was calculated to be significantly higher in the ABG group when compared to all other groups. In contrast to the total bone volume, the external bone formation (region 3) was higher in both cell groups and the scaffold only group than in the ABG group (Fig. 17).

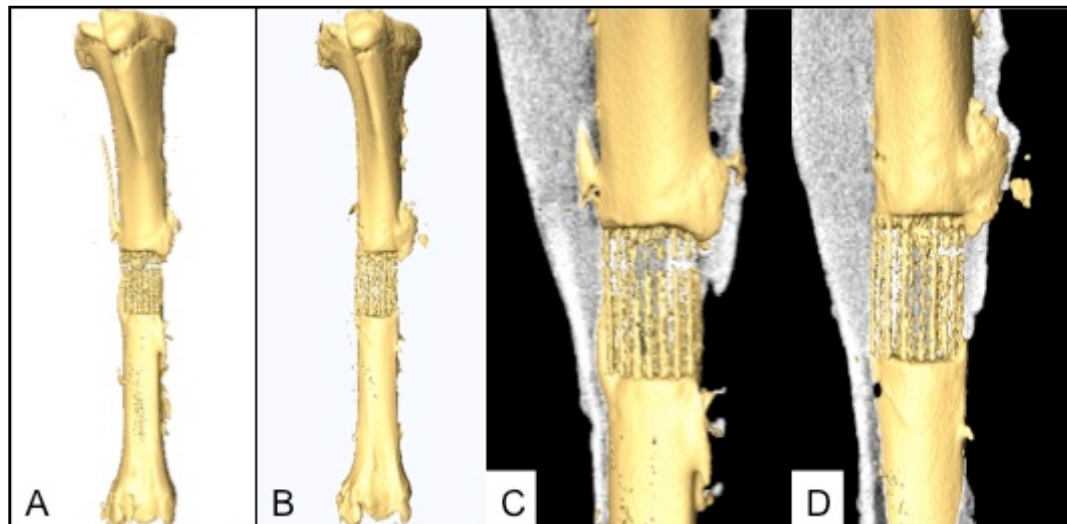


Figure 15: Representative 3D CT data reconstructions (AMIRA 5.2.2) after 12 weeks of critical segmental bone defects, which were treated with an mPCL-TCP scaffold seeded with autologous MPC's (A) and an mPCL-TCP scaffold seeded with allogenic MPC's (B). A 3D reconstruction with a threshold of 300 combined with a 2D reconstruction using a lower threshold to show the soft tissue and the scaffold showed a similar bone formation within the scaffold in the autologous cell group (C) and the allogenic cell group (D).

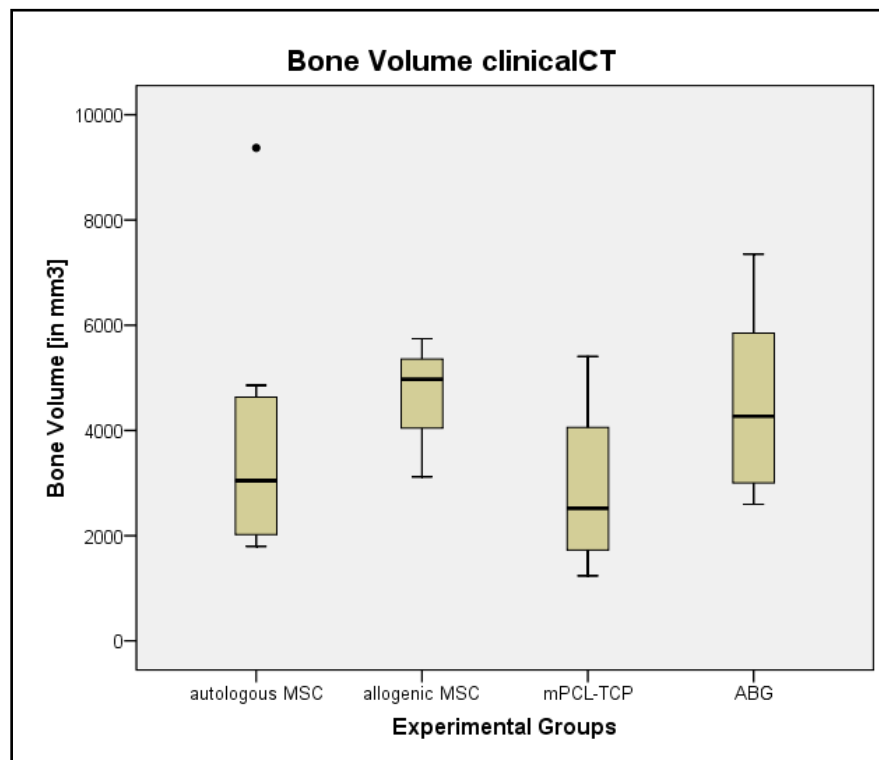


Figure 16: Box plot demonstrating the median \pm 1st and 3rd quartile. The figure illustrates the total bone volume (BV) after 12 weeks. Error bars represent minimum and maximum values. The cell groups showed more bone formation compared to the scaffold only group.

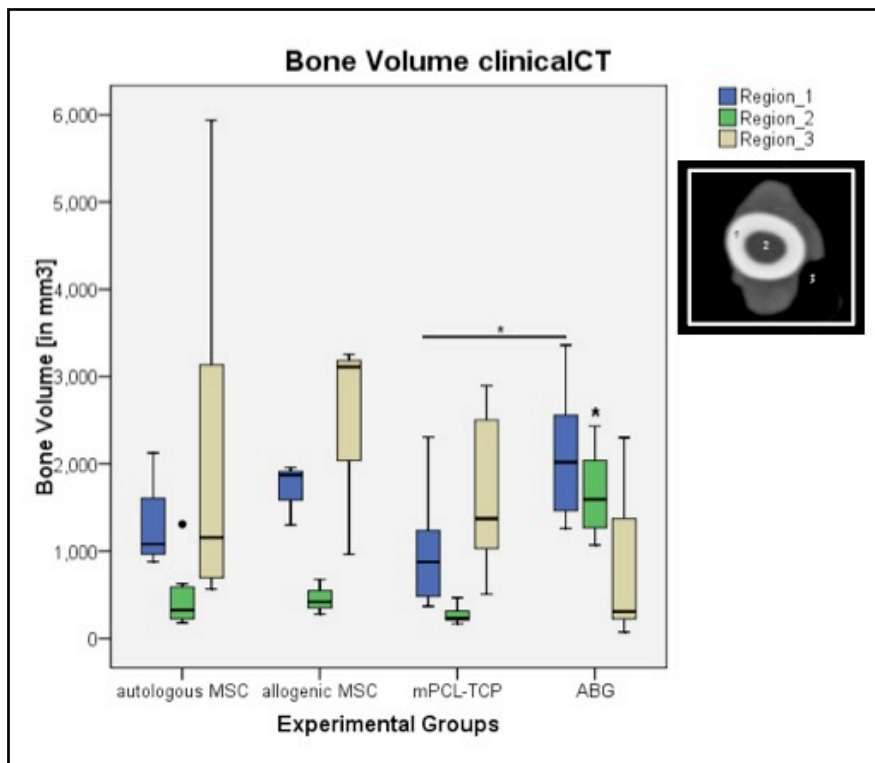


Figure 17: Box plot (the median \pm 1st and 3rd quartile) demonstrating the bone volume formed within the three regions of interest, the cortical region (region 1), the bone marrow region (region 2), and the external region (region 3) after 12 weeks. Error bars represent minimum and maximum values. Asterisks indicate statistical significance. Bone formation in region 2, the marrow region, was calculated to be significantly higher in the ABG group when compare to all other groups.

3.3.6. Biomechanical testing

Biomechanical testing was performed on all the specimens from the cell groups and compared with the results from previous experiments of our group⁷⁸ (Fig. 18-23). Biomechanical testing revealed an equal torsional stiffness (TS) for the autologous and the allogenic cell groups and slightly higher values for the torsional moment (TM) for the autologous group. Both cell groups showed a higher torsional moment, but a comparable torsional stiffness compared with the scaffold only group. The samples of the ABG group showed the highest values for torsional moment and torsional stiffness,

but they reached just about 15 to 20%, when compared to the contralateral non-operated side. No significant differences were found between all the groups at the biomechanical testing.

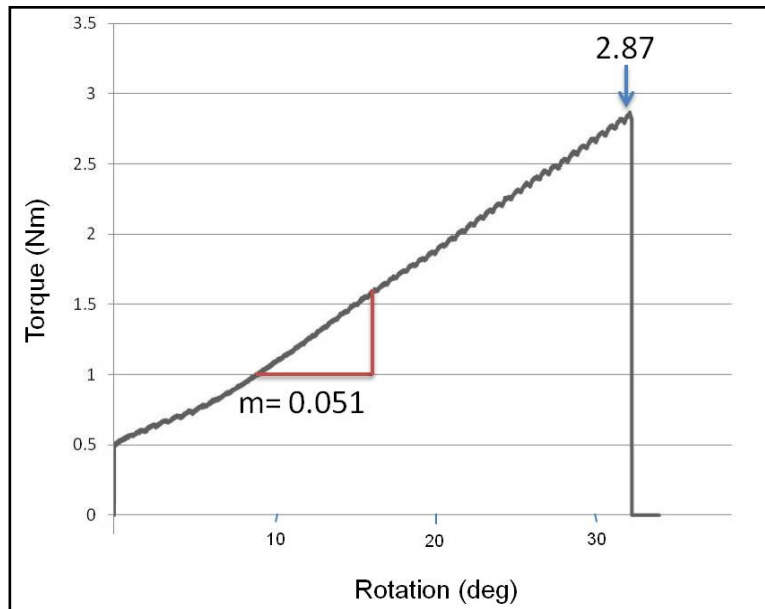


Figure 18: The torsional stiffness of the operated tibiae were calculated from the slope of the torque-angular displacement curves. The ultimate torsional moment was the peak value of the curve.

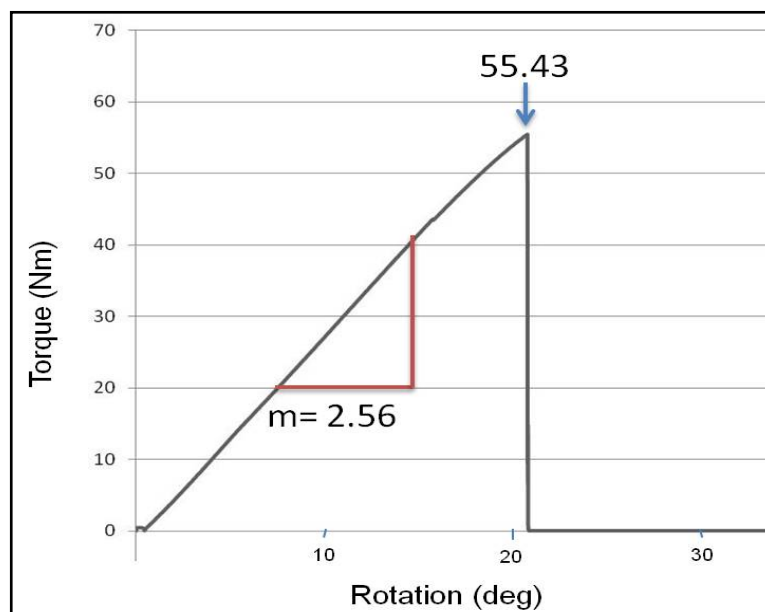


Figure 19: The torsional stiffness of the contralateral tibiae were calculated from the slope of the torque-angular displacement curves. The ultimate torsional moment was the peak value of the curve.

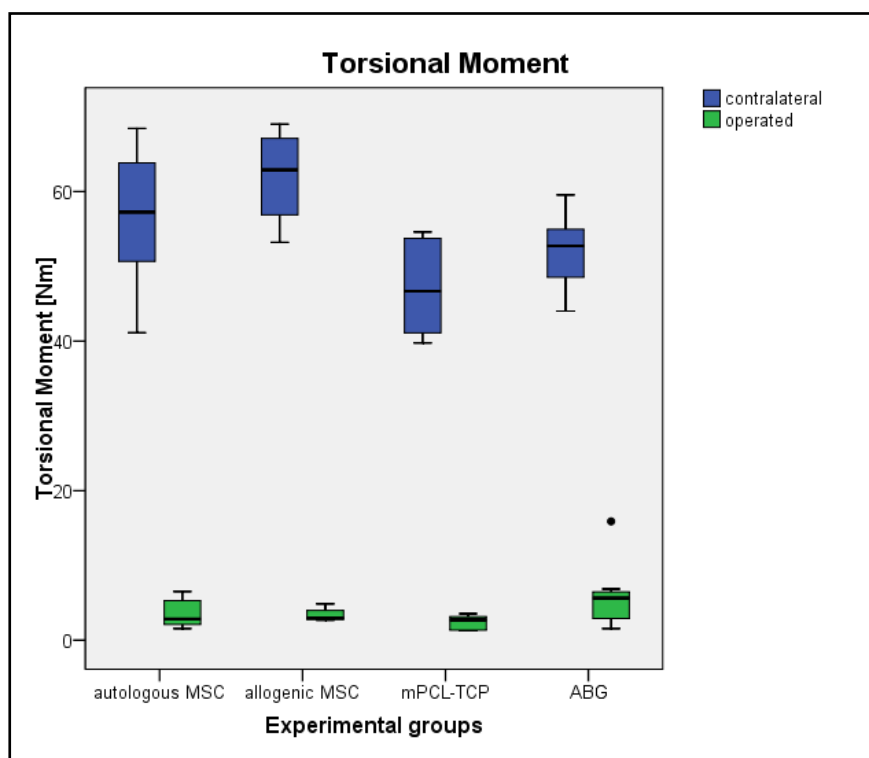


Figure 20: Results of the biomechanical testing (torsional moment) after 3 months. Box plots demonstrating median values \pm 1st and 3rd quartile of torsional moment of the operated (green) and the contralateral tibia (blue).

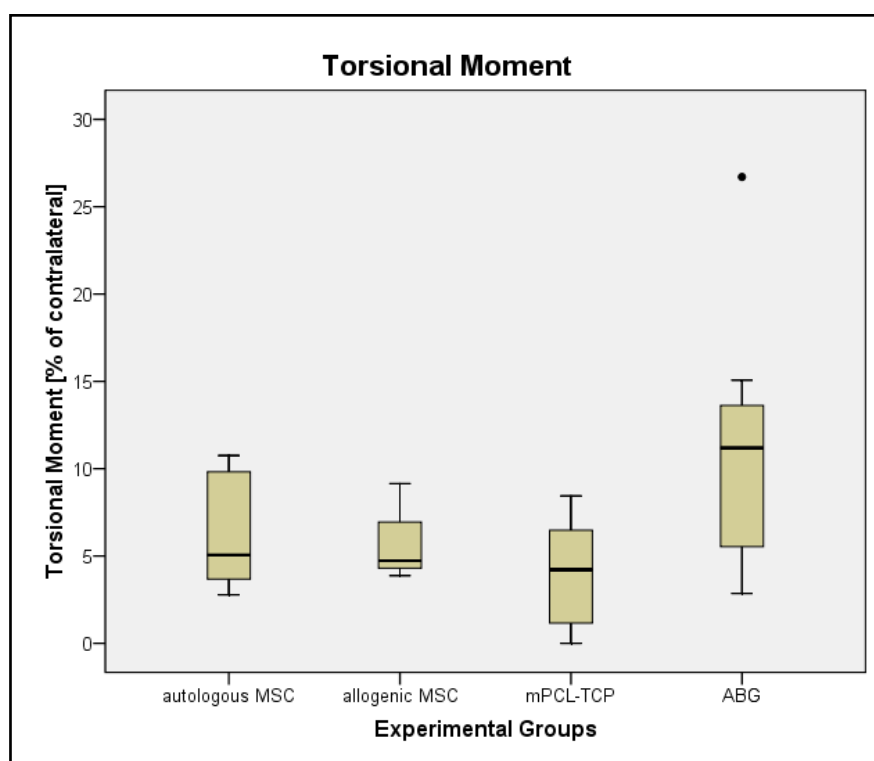


Figure 21: Results of the biomechanical testing (torsional moment) after 3 months. Box plots demonstrating median values \pm 1st and 3rd quartile of torsional moment in relation to the contralateral tibia. Error bars represent maximum and minimum values. Asterisks indicate statistical significance.

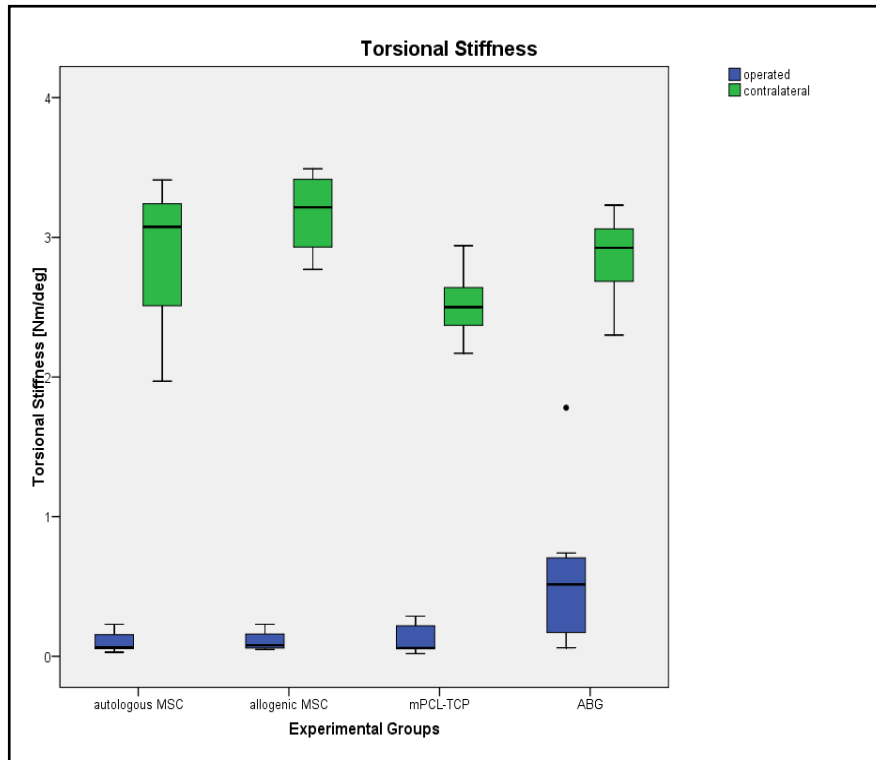


Figure 22: Results of the biomechanical testing (torsional stiffness) after 3 months. Box plots demonstrating median values \pm 1st and 3rd quartile of torsional stiffness of the operated (blue) and the contralateral tibia (green).

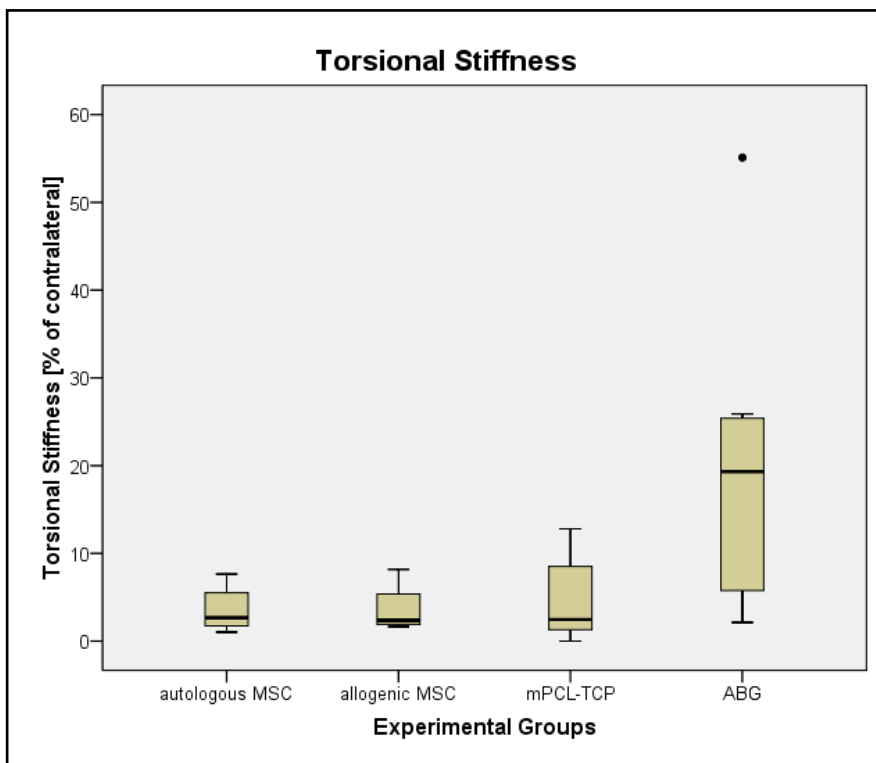


Figure 23: Results of the biomechanical testing (torsional stiffness) after 3 months. Box plots demonstrating median values \pm 1st and 3rd quartile of torsional stiffness in relation to the contralateral tibia. Error bars represent maximum and minimum values. Asterisks indicate statistical significance.

3.3.7. Computed tomography (microCT)

MicroCT analysis confirmed the trend of the results from the clinical CT scan regarding union rates and the amount of new bone formation. The differences in the absolute values were caused by the different scanning resolution of both methods. In the 3D reconstructions, both cell groups showed new bone formation mainly at the opposite site of the plate (Fig. 24 A/B). The 2D reconstruction showed additionally bone formation within the scaffold (Fig. 24 C/D). In both cell groups, the mean values of newly formed bone were higher compared to the scaffold only group. Highest median values of newly formed bone were found for the ABG group, which were significantly higher when compared to the scaffold only group, but without significant differences compared to both cell groups (Fig. 25). In all samples, newly formed bone was still less compared to the amounts determined for the same anatomic level of the contralateral hind limbs (Fig. 26). The amount of new bone was evenly distributed throughout the proximal, middle and distal third of the defect although a tendency towards higher amounts in the proximal defect third was observed (Fig. 27). The mineral density of the newly formed, woven bone or tissue was found to be homogenous in the different experimental groups. Notably, tissue mineral density values were significantly lower than those determined for the compact bone of the contralateral healthy tibiae (Fig. 28).

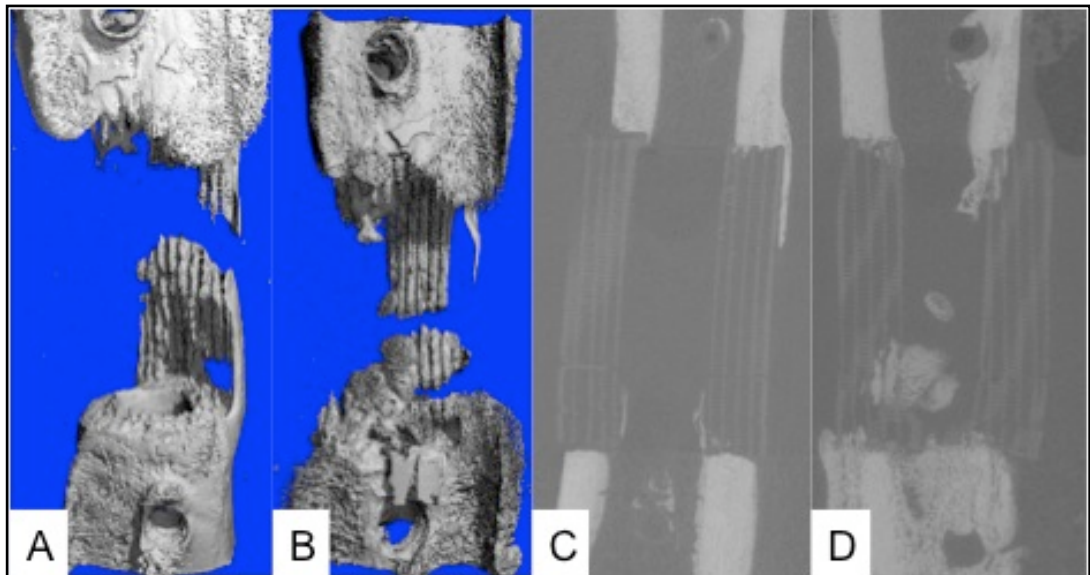


Figure 24: Representative 3D reconstructions and 2D sections of microCT scans of 3 cm tibial defects 12 weeks after surgery of the autologous cell group (A/C) and the allogenic cell group (B/D).

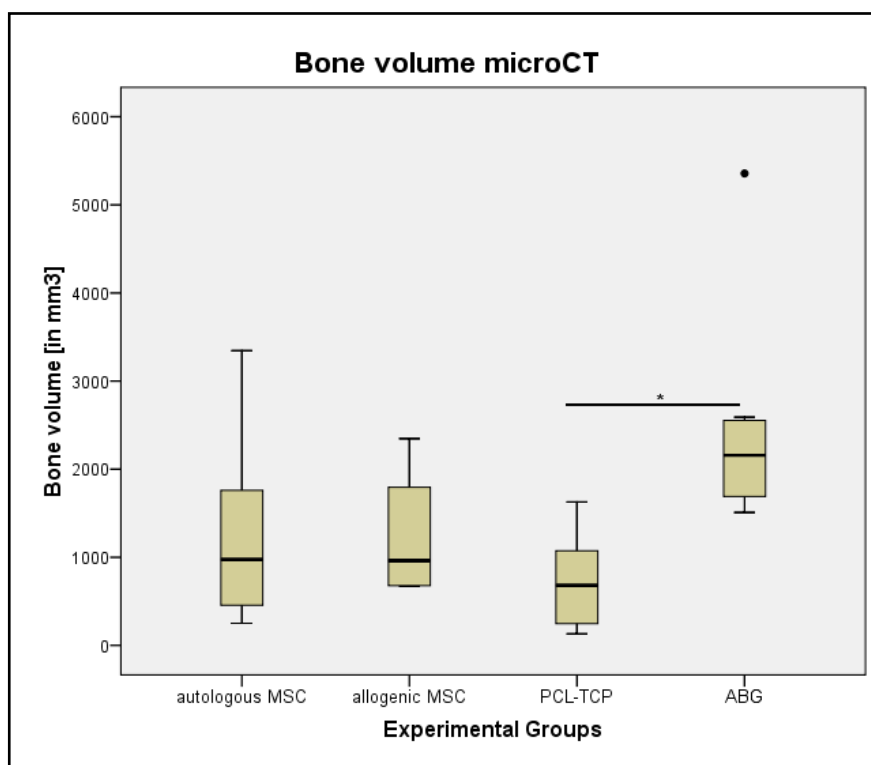


Figure 25: Box plot demonstrating median amounts of newly formed bone \pm 1st and 3rd quartile within the 3 cm defects 12 weeks after surgery. Error bars represent maximum and minimum values. Bars with asterisks indicate statistical significance.

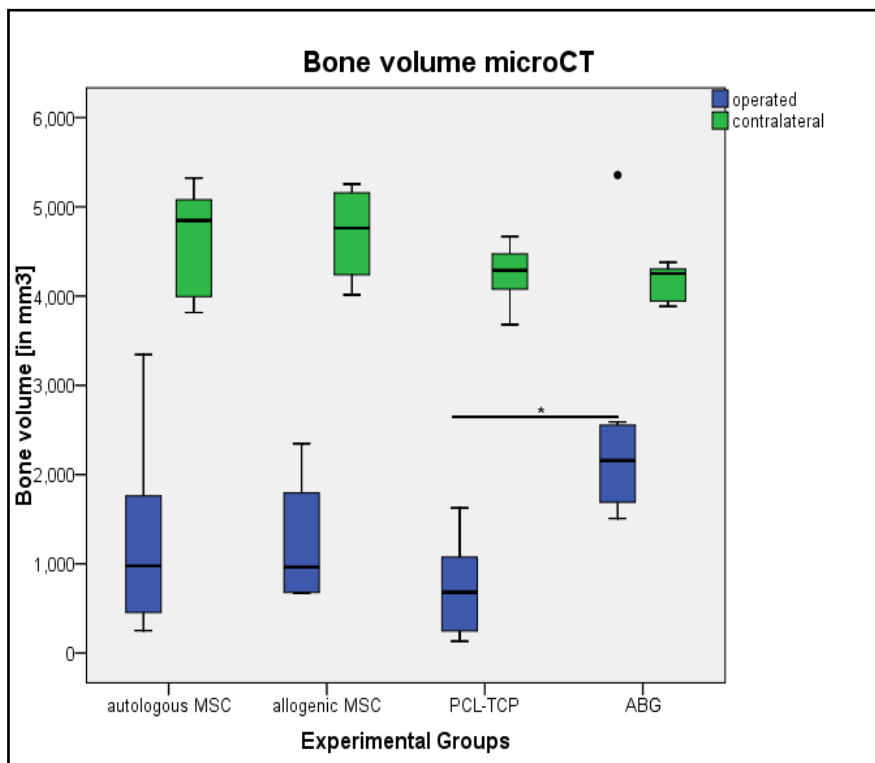


Figure 26: The blue box plots are demonstrating median amounts of newly formed bone \pm 1st and 3rd quartile 12 weeks after surgery of the operated site, in contrast to the same anatomic level of the contralateral hind limbs. Error bars represent maximum and minimum values. Bars with asterisks indicate statistical significance.

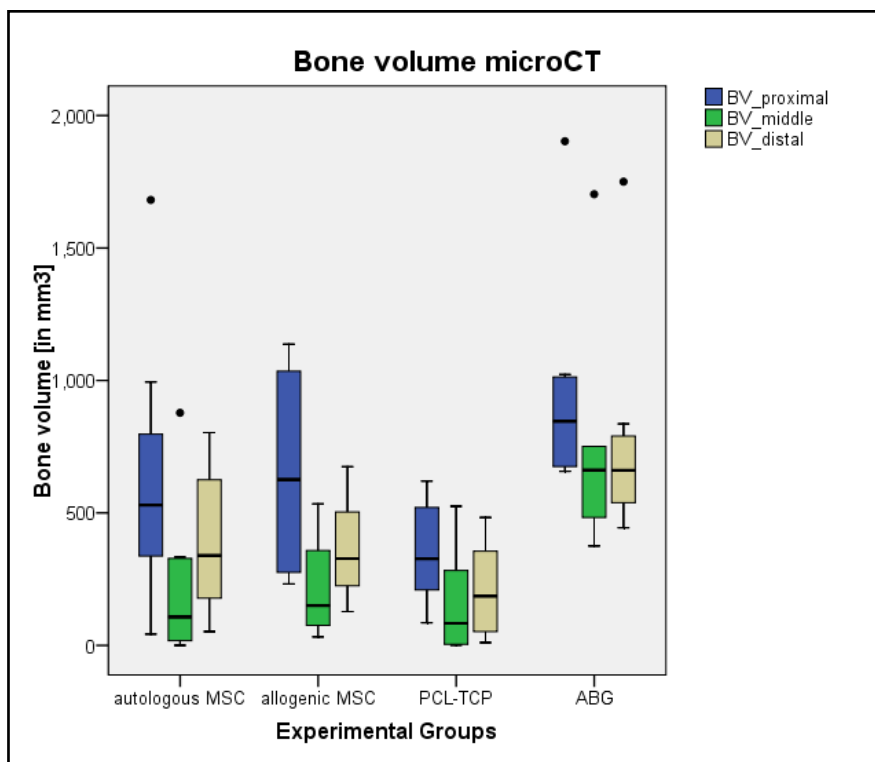


Figure 27: Box plot demonstrating the distribution of newly formed bone in mm³ divided in three equal parts (proximal, middle, distal) of regenerated defects. A tendency towards higher amounts of bone in the proximal defect third was observed throughout the different experimental groups.

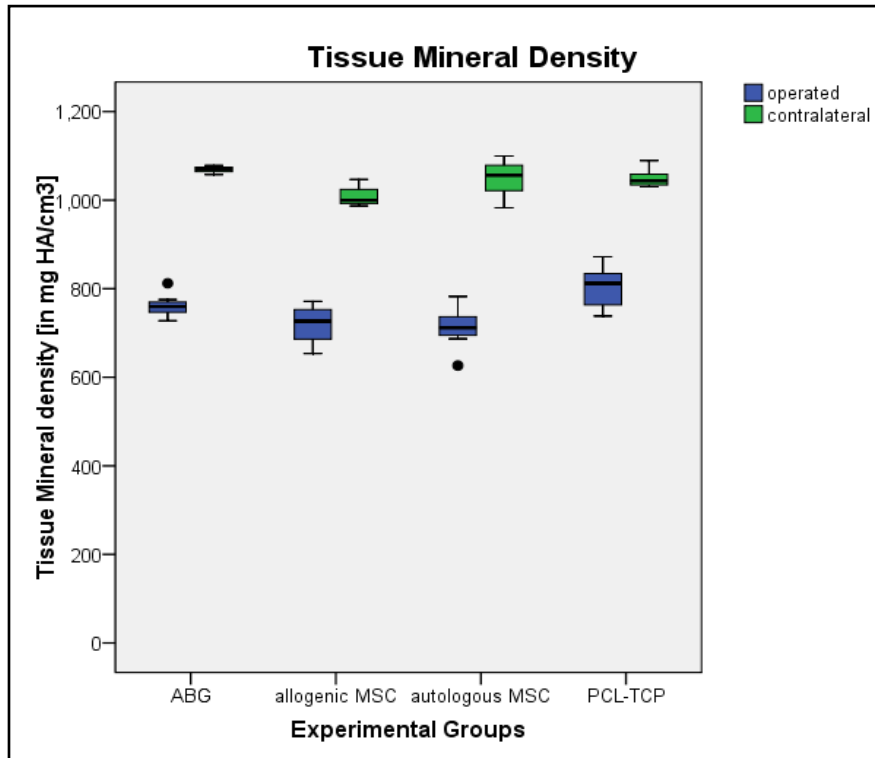


Figure 28: Box plot demonstrating median values for tissue mineral density within the defects \pm 1st and 3rd quartile 12 weeks after surgery. Tissue density did not differ significantly between the experimental groups. Tissue density in the defect zones was however only about 70% of that determined for the compact bone of the contralateral limb. Error bars represent maximum and minimum values.

3.3.8. Histology/Immunohistochemistry

The macroscopic overview of the implanted scaffolds before processing for histology showed a good integration of the scaffold to the host bone in all animals (Fig. 29 A). The mPCL-TCP scaffold was still in place and had not resorbed. Histological examinations of decalcified samples were performed after 12 weeks. Representative H&E staining of both cell groups demonstrated a good integration of the scaffold to the host bone on the proximal as well as the distal side (Fig. 29 B, C, F, G). Notably, new bone formation was seen in many pores within the mPCL-TCP scaffolds in both

cell groups (the scaffold itself is revealed in the histology slices as empty circular “holes” and “bars” where the scaffold struts used to reside, due to the dissolution of the mPCL implant by xylene during processing) (Fig. 29 B-G).

Undecalcified sections (MMA resin) were stained with von Kossa/McNeal's to identify new mineral deposition. Both cell groups showed mineral deposition (black stain) within the defect (Fig. 30 B, G). These results confirm our microCT and clinicalCT evaluation and demonstrate a limited amount of bone tissue growing into the whole scaffold area. Furthermore, the large overview pictures of the Goldner's Trichrome staining demonstrate the presence of collagen fibres in the scaffold and surroundings (Fig. 30 A, F). New bone formation is traditionally accompanied by the expression of bone specific proteins within the extracellular matrix surrounding the osteoblasts, this may be detected using immunohistochemistry. As an early non-specific marker of osteoblastic differentiation during mineralisation, type I collagen showed a similar intensity in both cell groups (Fig. 30 D, I). Immunohistochemical staining for osteocalcin also demonstrated similar expression levels with a high expression of this late osteogenic marker localised around the scaffolds struts (labelled “s”) (Fig. 30 E, K). The staining to detect the BrdU labelled cells did not reveal any evidence of survival of the donor cells (data not shown).

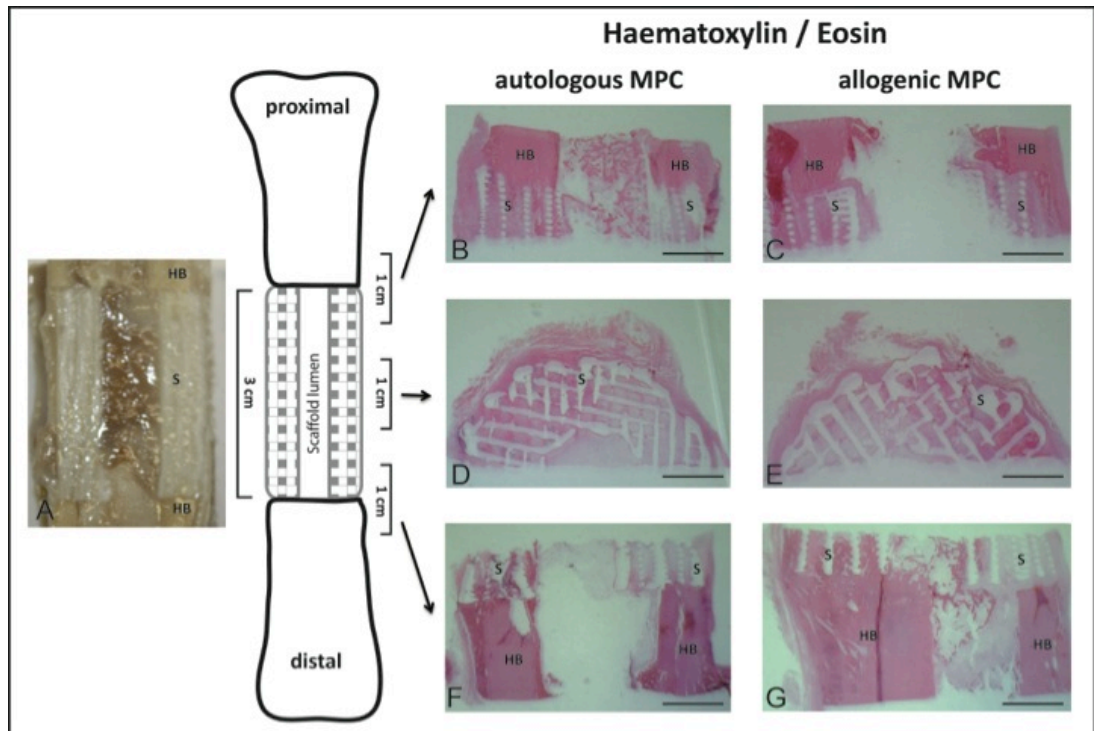


Figure 29: Macroscopic overview of the scaffold/cell constructs within the defect after explantation after 12 weeks (A). Representative H&E staining of both cell groups of the proximal (B, C) (longitudinal sections), middle (D, E) (transverse sections) and distal (F, G) (longitudinal sections) parts of the defect showed a good integration of the scaffold (S) as well as a good bonding of the regenerated bone to the host bone (HB), (B-G; bar=0.5cm). The solvents used during the preparation of the histological sections resulted in the mPCL–TCP scaffold material being dissolved during embedding. Hence mPCL–TCP struts (S) are represented in histological sections as empty “holes” of similar geometry.

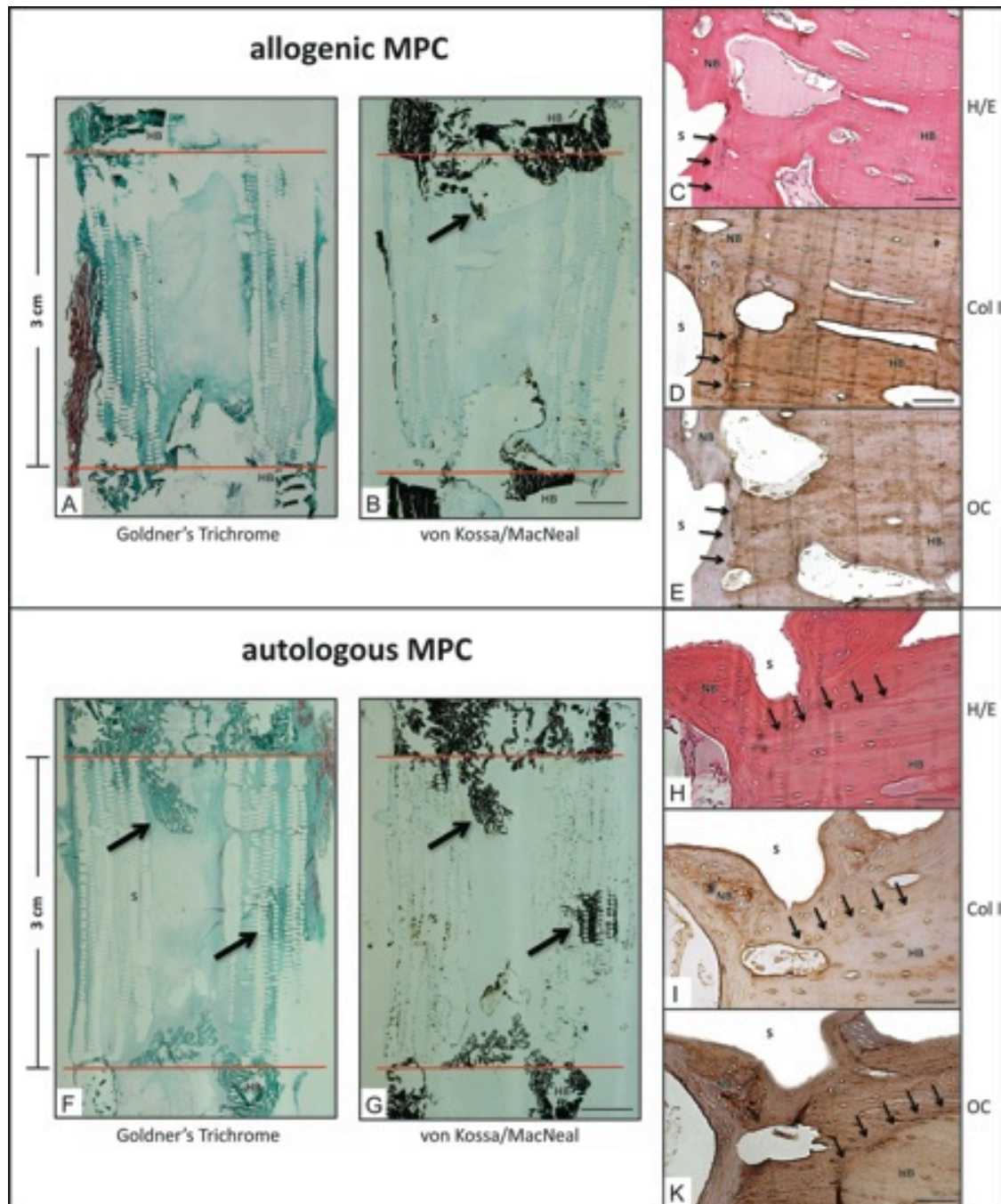


Figure 30: Overview pictures of the undecalcified resin-embedded samples after 12 weeks, sectioned and stained with von Kossa/McNeal's (B, G; bar=0,5cm) and Goldner's Trichrome (A, F) demonstrated the mineralized tissue within collagen fibres (black arrows). Decalcified samples were stained with H&E (C, H), collagen type I antibody (D, I) and osteocalcin antibody (E, K); (bar in C-E, H-K=100μm). Representative stainings of both cell groups showed a good integration of the scaffold (scaffold strut labelled s) as well as a good bonding of the regenerated bone (NB) to the host bone (HB)(black arrows in E, H, I and K). The solvents used during the preparation of the histological sections resulted in the mPCL–TCP scaffold material being dissolved during embedding. Hence mPCL–TCP struts (S) are represented in histological sections as voids of similar geometry.

3.4. Discussion

Given the demographic challenge of an ageing population, the development of strategies to exploit the potential of stem and progenitor cells to augment bone formation to replace or restore the function of traumatized, diseased, or degenerated bone is a major clinical and socioeconomic need.

The use of allogenic cells for clinical therapy is already established and in routine use in other areas of medicine such as oncology and a large number of clinical trials are currently performed in cardiology^{268,269}. These studies suggested that hMSCs could be used therapeutically as allogeneic, “universal cells.” To support this suggestion, it has been further documented that culture-expanded hMSCs do not have MHC class I cell surface markers, but rather only MHC class II and no co-stimulator molecules²⁷⁰. Thus, hMSCs cannot be antigen-presenting cells and should be imperceptible to the host’s immune system. It is important to note that in all of the clinical usages of human adult marrow-derived, culture-expanded MSCs, whether autologous or allogeneic, no adverse events have been recorded²⁷¹.

The results of this study demonstrated the positive effect of allogenic bone marrow-derived mesenchymal progenitor cells (MPC’s) on scaffold-based bone regeneration. In addition to their osteogenic potential, it has been shown that MPCs are immunologically privileged making them highly appropriate for the use in allogenic cell transplantation concepts, and indeed we observed no immune response in a preclinical animal model. Furthermore, Niemeyer et al. demonstrated that MPC retain their

immunological properties after osteogenic induction *in vitro*²⁷². The allogenic application of MPCs showed similar results for bone regeneration compared to the autologous cell group, with all the advantage of an allogenic cell source (easy to access and high abundance compared to autologous cells). Our results are in line with observations from Guo et al. who described that allogenic mesenchymal progenitor cells raised a minimal immunological reaction only in the early stages after implantation in a study in mini-pigs²⁷³. After the transplantation, the sheep were monitored by clinical examination and taking blood samples. We did not observe any general foreign body reaction or cell rejection or any different cellular reactions compared to the autologous group in the histological assessment throughout the defect, which demonstrated, once more, the low immunological activity of allogenic bone marrow derived cells. While the limited availability and the donor site morbidity of autologous bone grafts is often discussed and stated as a major disadvantage of this technique, the use of an allogenic cell source with comparable regeneration potential as a potential off-the-shelf-product would open new routine therapeutic potentials for regeneration of large bone defects^{274,275}.

Niemeyer et al. used human MPCs to assess bone regeneration in a critical sized defect of the sheep tibia and compared the regenerative potential with autologous ovine MPCs²⁷⁶. The autologous MPCs demonstrated better bone formation compared to the human MPCs and unloaded matrices in the histological and radiological evaluation. However, the bone regeneration was evaluated based on two dimensional imaging procedures and the study lacked any biomechanical testing, which would provide a greater depth of

understanding of the characteristics and functionality of the regenerated bone. Field et al. evaluated the efficacy of allogenic mesenchymal progenitor cells for the repair of an ovine tibial segmental defect²⁶². They reported a higher osteogenic regeneration potential of the allogenic group compared to the scaffold only group with better biomechanical properties and more bone formation. But, studies by Field et al. and Niemeyer et al. used different fixation methods (double plate vs. intramedullary nail) and different experimental protocols, which renders direct comparison of these findings difficult. Using a medullary nail for example will result in blocking the medullary cavity of the tibia, which might reduce the regeneration potential in this area. Furthermore, different fixation methods result in different biomechanical impacts, such as more flexibility and a central loading (nail) compared to a much stiffer double plate fixation. Our biomechanical analysis of the DCP-plate fixation showed, under static loading conditions of up to 500 N, a minimal displacement of the scaffold of less than 1%. Conclusively, the DCP results in a stable fixation. From an ethical as well as an economical point of view, it would be desirable to be able to directly compare the potential of different tissue engineering strategies to lower the costs and the number of animals and to realise the translation of novel concepts from research to clinical practice. Therefore, the bone tissue engineering community should advocate the combination of radiological, biomechanical, histological and immunohistochemical evaluations as necessary methodologies for an efficient and robust analysis of bone engineering strategies²⁷⁷.

As bone tissue engineering has become ever more prevalent over recent years, evaluating the different strategies with respect to potential clinical application in humans requires *in vitro* testing and small animal models and importantly, in the final stages, the use of standardised large animal models which are imperative for rigorous preclinical evaluation²⁷⁸. Thus, large preclinical animal models with comparable body weight, long bone dimensions, similar mineral composition, equivalent remodelling rates as well as established and standardised evaluation processes are essential to make sufficient predictions about the potential clinical success or failure of new bone tissue engineering strategies^{76,77}. Over the last decade, the sheep has become a very important and useful model to address research problems and holds specific advantages compared to other large animal models (e.g. pig, dog) such as long bone dimensions, ease of handling, non aggression nature, and the ability to keep them in large numbers at relatively low costs. However, it is important to note that secondary bone remodelling is only seen in older sheep making them comparable to human bones²⁷⁹.

Our group has established an ovine critical-sized segmental tibial defect model to address different bone tissue engineering approaches by testing combinations of growth factors, scaffolds and different cell types^{77,266}. Compared to studies by other groups, this current model is a particularly challenging model using older sheep which are on the one hand more relevant to human bone but on the other hand have a lower general health status compared to younger animals. Some other groups only resect the periosteum on the proximal and the distal bone, however, we have shown in a preliminary study, that the periosteum on the dorsal side between the tibia

and the muscles which is closed to the vascular bundle (see back arrow Fig. 4F) contains a significant regeneration potential based on the MPCs from the cambium layer²⁶⁶. To our knowledge, we are the first group who developed a large segmental tibial defect model, which also includes the removal of this part of the periosteum, which represents 30-40% of the whole volume of the periosteum within the defect. As a result of the complete removal of the periosteum and the age-related compromised regenerative capacity, it is not surprising that we revealed lower biomechanical stability (TM 10-15% and TS 20-25% of contralateral tibia) after three months even in the ABG group compared to other research groups who even present significantly higher results in their control groups^{280,281}. This demonstrates once again the importance of standardised large animal models with validated experimental protocols to compare outcomes of each different bone engineering approach. This is a *condition sine qua non* to move concepts from bench to bedside.

The present study showed effectively that allogenic MPCs can be safely used in combination with a mPCL-TCP scaffold for bone regeneration in a critical sized bone defect in a sheep model. None of the animals of the study showed a rejection of the TECs or a foreign body reaction in form of a fibrous encapsulation. The allogenic and the autologous cell groups showed comparable results with respect to biomechanical properties and new bone formation. The biomechanical evaluation showed a small difference between the cell groups and the scaffold-only group, whereas the bone volume analysis using clinicalCT and microCT showed larger differences between these groups. This is likely due to the short time frame (three months) we chose for our experiment to terminate. We detected more bone volume in

both cell groups compared to the empty scaffold groups, which demonstrated that autologous as well as allogenic cell transplantation leads to enhanced bone formation in a critical sized segmental defect model. But within the three months time period, the bone regeneration was still in its early progress phase and most of the defects were not completely bridged which resulted in low biomechanical test results. Most other studies using a critical sized segmental tibial defect in a large animal had longer time frames for their experiments, which logically resulted in more bone formation and higher biomechanical stability²⁶². As we were specifically interested in considering the immune response and the safety of delivering allogenic MPCs at this stage of our research we intentionally chose an early time point.

A common practice to improve the biological competence of osteoconductive scaffolds is via incorporation of osteogenic stimuli such as bone morphogenetic proteins (BMP) and/or vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF). Platelet rich plasma (PRP) consists of several growth factors including significant amounts of VEGF and PDGF and has been in clinical use for many years in the dental surgery field. However the benefits of PRP are still ambiguous regarding large bone defect regeneration in orthopaedics, whereas the positive effect of BMPs for bone regeneration has been shown extensively in several small and large animal models as well as in the clinic^{89,93-96}.

Because of the similarity to clinically used fibrin glue, PRP converts from a liquid state into a fibrin hydrogel in the solid state after being activated by thrombin and therefore it is used in several studies as a highly biocompatible cell-loading vehicle⁹⁷. We could demonstrate that the scaffold-PRP construct

used in our study for large bone defects didn't have significant effects regarding bone regeneration when compared to the scaffold only group (unpublished data). But it can be used as a very effective cell delivery vehicle in a scaffold design with a channel-like architecture with large pores and large pore interconnections which allow full vascularization in a 3 cm segmental bone defect.

The evaluation of the bone volume using both clinical- and micro-CT scanning showed slight differences in the raw data (absolute values) due to the different resolutions as well as the different phantoms used for calibration; however the trend was the same. The experimental groups all showed a comparable tendency with respect to the contralateral tibia of the animals (Fig. 7 F). Keeping in mind the concept of translational research which might result in a later clinical application, the assessment of the correlation between clinicalCT scanning and microCT scanning becomes important, especially for future interpretation of clinicalCT scanning results from large bone defects in humans, as these may be directly related to the CT-results of the large animal studies. Beside the CT-scanning, which is essential for the 3D-reconstruction overview and analysis of the bone volumes, the histological evaluation plays an important role for a detailed evaluation on a cell-based level. Therefore, both evaluation methods (CT-scanning and histology) are absolutely required to perform a reliable study to analyse different tissue engineering approaches for bone regeneration.

The use of cell-based tissue engineering strategies is often compromised due to a low survival rate of the implanted cells. To prove the effect of the transplanted cells, different labelling methods are available to demonstrate

the survival of the transplanted cells at the end of the experiment²⁸². Among these, BrdU-labelling is a straightforward method of cell labelling showing good results in small animal models. However, despite the potential shown using small animal models, we were not able to detect any BrdU-labelled cells in the large bone defect model²⁸³. This may be due to labelling the cells in passage 1 and using the cells of passage 3-4 for the *in vivo* experiments, because of the high amount of cells required for the experiment, hence the proliferation of the cells may have led to a loss of signal. Li et al. reported a decreasing of the labelling signal of BrdU over time, describing that the percentage of BrdU-positive cells decreased from 94% in passage 0 down to 18% in passage 2²⁸². Therefore, for the use in large segmental bone defects such as our sheep model, another labelling method would be more appropriate to proof the survival of the cells^{284,285}.

Furthermore, a detailed and critical understanding of using allogenic cells for bone tissue engineering would be desirable, but can't be easily achieved from a histological perspective when using ovine cells, because of a lack of specific ovine antibodies. These precise questions could be answered by using human cells in a xenogenic transplantation setting, but as Niemeyer et al. demonstrated, human MPCs led to reduced bone formation in a ovine defect model compared to the use of allogenic cells²⁷⁶.

3.5. Conclusion

Although MPCs can differentiate into various phenotypes of mature cells, their intrinsic capacity to secrete cytokines and growth factors at sites of

tissue injury and inflammation contribute significantly to their therapeutic capacity. We assume that the production of these trophic mediators is defined by their *in vivo* location, niche, and severity of injury. The present study showed promising data using an allogenic compared to autologous cell source for regeneration of critical sized segmental bone defects in a large animal model. The use of allogenic or autologous cells combined with an mPCL-TCP scaffold showed no differences in their bone regeneration potential as demonstrated by radiological, histological and biomechanical results. We furthermore detected no adverse immunological response. In the future, the successful translation of allogenic cell transplantation into clinical practice could provide beneficial treatment alternatives for challenging bone defects.

Chapter IV – Assessment of the regenerative potential of allogenic osteoblasts versus allogenic mesenchymal progenitor cells in the reconstruction of ovine critical sized segmental tibial bone defects

4.1. Introduction

Mesenchymal progenitor cells (MPCs) and osteoblast cells (OB) have been considered in cell-based tissue engineering strategies. Osteoblast cells can be isolated from different bones of the axial skeleton²⁶¹. Looking to the embryological development of the bones, there are differences between the bones of the axial skeleton and the orofacial bones²⁸⁶.

In orofacial surgery, autologous bone grafts used to stimulate new bone formation at sites of orofacial osseous defects are commonly obtained from several donor sites including orofacial, axial and appendicular bones. Bridging orofacial defects with grafts obtained from an orofacial donor sites are usually more successful than those from non-orofacial sites, indicating anatomic skeletal site-specific differences affecting graft integration^{287,288}. This clinical observation, along with the fact that many bone abnormalities (such as cherubism²⁸⁹ and hyperparathyroid jaw tumor syndrome²⁹⁰) are limited to craniofacial bones, suggests that there are differences in bone

metabolism in orofacial and axial bones. These differences should be dictated by site-specificity of embryological progenitor cells and osteogenic properties of resident multipotent bone marrow stromal cells. The embryological development of the complex array of bones and cartilage in the craniofacial skeleton are different from those occurring in other body sites²⁹¹.

The orofacial bones are formed exclusively by neural crest cells, while axial bones are formed by mesoderm²⁹². Neither chondrocytes nor osteocytes, however, are unique to neural crest cells; both skeletal cell types also arise from the mesodermal cells, which form the axial, appendicular and rib skeleton. Neural crest cells are marked by expression of a number of genes, including members of the *MSX*, *slug/snail*, *Zic*, *Pax-3/7*, and the *Distalless* gene family. The neural crest, and the subsequent development of the craniofacial skeleton, represents an important evolutionary innovation. These clinical, laboratory and developmental differences imply the existence of site-specific properties of progenitor cells in bone marrow. Therefore, the cells isolated from these bones have different origins and ways of bone formation. Osteoblasts, isolated from orofacial bones form bone via intramembranous bone formation, whereas osteoblasts, isolated from axial bones form bone via endochondral bone formation (Fig. 31)²⁹³.

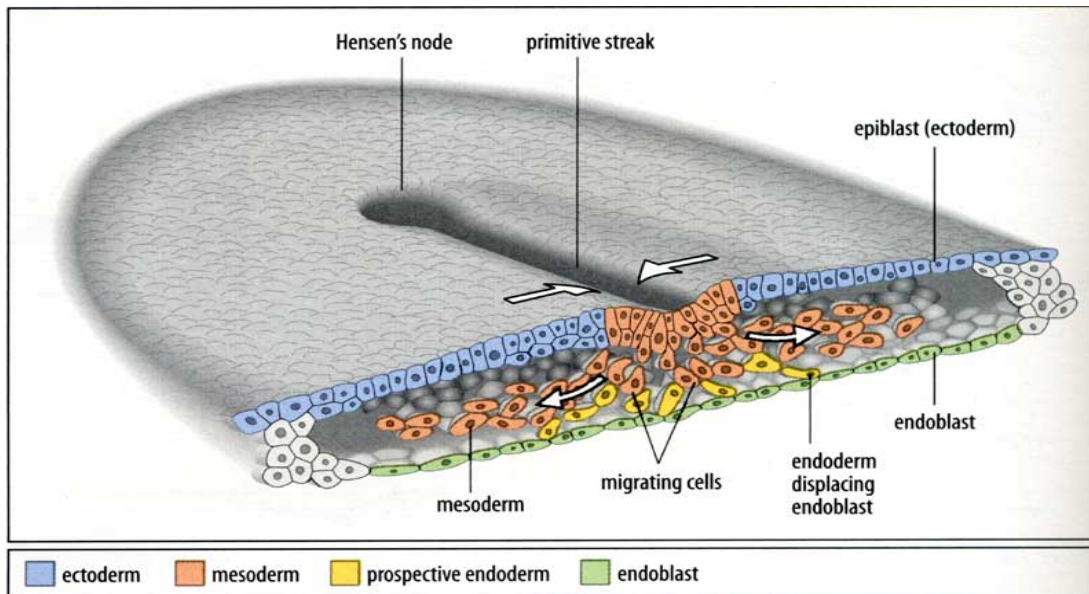


Figure 31: Embryological development of the human skeleton. The orofacial bones are formed by ectoderm (blue), whereas the axial bones are formed by mesoderm (orange)(Figure from Wolpert et al.)²⁷⁸.

During further *in vitro* experiments, our group detected significant differences in the osteogenic potential (production of extracellular matrix, bone volume, osteogenic differentiation) of osteoblasts from orofacial bones (mOB), osteoblasts from axial bones (tOB) and mesenchymal progenitor cells (MPC's)²⁹⁴. We could demonstrate, that mesenchymal progenitor cells show a higher osteogenic potential *in vitro* compared to differentiated cells (osteoblasts). Surprisingly, the *in vivo* experiments show a higher osteogenic potential of osteoblasts compared to mesenchymal progenitor cells in an ectopic setting, when transplanted subcutaneously in mice²⁷⁹.

Cell-based strategies in tissue engineering approaches for bone tissue regeneration include implantation of cell-seeded three-dimensional scaffolds in the defect. These cells should have a high osteogenic regenerative potential. The supply of autologous mesenchymal progenitor cells and osteoblasts is often limited and the preoperative procedure for isolation, augmentation and differentiation is time consuming. But their special

immunological characteristics suggest that MPC's could be used in non-autologous applications^{257,258}. Allogenic cell transplantation is a common therapeutic option and is in routine clinical use in the field of oncology^{263,264}. In further *in vitro* experiments by our group, we found a similar expression of surface markers between mesenchymal stem cells and osteoblasts. Hence, a non-autologous use of both cell types should be possible²⁷⁸. Translating the idea of allogenic cell-transplantation from oncology to orthopaedics could offer a new opportunity for the availability of mesenchymal stem cells for regenerative medicine and a clinical application in orthopaedic and trauma surgery.

The combination of different cells, biomaterials, and different kinds of growth factors and a combination thereof is a complex process with interdependent sets of variables. Therefore, the different developments must be monitored and tested consequently *in vitro* and *in vivo* before translating new treatment concepts into clinical practice.

Based on the results of previous experiments, we hypothesized that osteoblasts (differentiated cells) show a higher osteogenic potential than mesenchymal progenitor cells in a scaffold-cell based bone tissue engineering concept for regeneration of a critical sized segmental defect in a large animal model. The aim of the current study was to assess and compare the regenerative potential of allogenic bone marrow mesenchymal progenitor cells (allogenic MPC's) with allogenic osteoblasts isolated from axial bones (allogenic tOB's – mesenchymal origin) and from orofacial bones (allogenic mOB's – neurocrestal origin) in combination with a mPCL-TCP scaffold in a critically sized segmental bone defect in sheep tibiae.

4.2. Material and Methods

4.2.1. Scaffold fabrication and preparation

The scaffolds used in this study were the same as used in the previous studies. For detailed information about the scaffold fabrication and preparation please see chapter III, part 3.2.

4.2.2. Animal study

Thirty-two male sheep (40-50 kilogram, age 7-8 years) were used in this project (Table 6). An Animal Ethics Approval Certificate has been obtained. Animal surgery was performed at the QUT Medical Engineering Research Facility, The Prince Charles Hospital, Chermside. There were 8 animals in each group and all were euthanized after 6 months.

The research plan for this study is structured as follows:	Group I	Group II	Group III	Group IV
	mPCL-TCP + PRP	mPCL-TCP + allogenic MPC	mPCL-TCP + allogenic mOB	mPCL-TCP + allogenic tOB
Total	8	8	8	8

Table 6: The research plan with details of the animal treatment groups. The time duration of this experiment was 6 months.

4.2.3. Preparation of platelet rich plasma

The method to produce platelet rich plasma (PRP) was described in detail in the previous chapter. For detailed information please see chapter III, part 3.2.3.

4.2.4. Animal Surgery

The anaesthetic procedures, the pre-operative treatment and the surgical procedures were already described in chapter III. For detailed information please see chapter III, part 3.2.4.

4.2.5. Experimental Groups

- Group I

The empty scaffold (mPCL-TCP) was loaded with a mixture of 250 µl of basal medium and 1ml of PRP and then seeded onto each collagen type I coated mPCL-TCP scaffolds. PRP was activated with thrombin and the cell scaffold constructs were implanted into the defect site and the wound closed in layers around the autograft.

- Group II

Allogenic ovine mesenchymal progenitor cells (allogenic MPC) were obtained from Merino sheep that were not included in this study (allogenic cells). Bone marrow aspirates were obtained from the iliac crest under general anaesthesia. Total bone marrow cells ($5-15 \times 10^6$ cells/ml) were plated at a density of $10-20 \times 10^6$ cells/cm² in complete medium consisting of low glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were subsequently plated at a density of 10^3 cells/cm². Two weeks before implantation, the medium was changed to an osteogenic media (DMEM, 10% FBS, 100 U/ml penicillin and 100 µg/ml

streptomycin, 10 µl/ml β-glycerophosphate, 1 µl/ml ascorbic acid and 1 µl/ml dexamethasone) to induce osteogenic differentiation. For 3D cultures, 35 x 10⁶ ovine MPC suspended in 250 µl of basal medium were mixed with 1ml of PRP and then seeded onto each collagen type I coated mPCL-TCP scaffolds. PRP was activated with thrombin and the cell scaffold constructs were implanted into the defect site and the wound closed in layers around the autograft.

- Group III

Allogenic ovine mandibular osteoblast (mOB) explants were obtained from Merino sheep that were not included in this study (allogenic cells). Compact bone samples were collected under sterile conditions from the mandibular under general anaesthesia with a special trephine drill (Ø 5mm), minced, washed with PBS and vortexed a minimum of 5 times. Bone samples were incubated with 10 ml 0.25% trypsin/EDTA for 3 min at 37°C, 5% CO₂. After trypsin inactivation with 10 ml low glucose Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum (FBS), samples were washed once again with PBS and transferred to 175 cm² tissue culture flasks. Samples were topped-up with 15 ml of DMEM containing 10% FBS and 1% penicillin/streptomycin. Outgrowth of osteoblasts was observed after 5-7 days. Cells were expanded to the second or third passage for following experiments. Two weeks before implantation, the medium was changed to an osteogenic media (DMEM, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 10 µl/ml β-glycerophosphate, 1 µl/ml ascorbic acid and 1 µl/ml dexamethasone) to induce osteogenic differentiation. For 3D cultures, 35 x

10^6 ovine mOB suspended in 250 μ l of basal medium were mixed with 1ml of PRP and then seeded onto each collagen type I coated mPCL-TCP scaffolds. PRP was activated with thrombin and the cell scaffold constructs were implanted into the defect site and the wound closed in layers around the autograft.

- Group IV

Allogenic ovine tibial osteoblast (tOB) explants were obtained from Merino sheep that were not included in this study (allogenic cells). Compact bone samples were collected under sterile conditions from the tibia under general anaesthesia with a special trephine drill (\varnothing 5mm), minced, washed with PBS and vortexed a minimum of 5 times. Bone samples were incubated with 10 ml 0.25% trypsin/EDTA for 3 min at 37°C, 5% CO₂. After trypsin inactivation with 10 ml low glucose Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum (FBS), samples were washed once again with PBS and transferred to 175 cm² tissue culture flasks. Samples were topped-up with 15 ml of DMEM containing 10% FBS and 1% penicillin/streptomycin. Outgrowth of osteoblasts was observed after 5-7 days. Cells were expanded to the second or third passage for following experiments. Two weeks before implantation, the medium was changed to an osteogenic media (DMEM, 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin, 10 μ l/ml β -glycerophosphate, 1 μ l/ml ascorbic acid and 1 μ l/ml dexamethasone) to induce osteogenic differentiation. For 3D cultures, 35×10^6 ovine tOB suspended in 250 μ l of basal medium were mixed with 1 ml of PRP and then seeded onto each collagen type I coated mPCL-TCP scaffolds. PRP was

activated with thrombin and the cell scaffold constructs were implanted into the defect site and the wound closed in layers around the autograft.

4.2.6. Blood analysis

All allogenic groups were monitored for six weeks after the procedure to assess any reaction to the implantation of the cells. Blood samples were taken from all animals of the allogenic group and from the animals of the scaffold only group as a control. Blood samples were taken on day 1,3,7,14,21 after the operation. At the same time the sheep were physically examined. A final physical examination was performed 6 weeks after surgery.

4.2.7. Radiography analysis

Throughout the study, x-rays were taken at 3 and 6 months, to determine the bridging time of the defect in the different experimental groups. Conventional x-ray analysis (3.2 mAs; 65kV) was performed in two standard planes (anterior-posterior and medial-lateral). At euthanasia, the gross morphology and mobility at host-graft junctions was clinically assessed and the findings carefully documented and photographed.

4.2.8. Biomechanical evaluation

To determine the integration of the scaffolds into the bone and the recovery of the biomechanical function of the affected tibias, biomechanical testing was performed after 6 months. The detailed procedure of biomechanical

testing was already described in chapter III. For detailed information please see chapter III, part 3.2.9.

4.2.9. Computed tomography (microCT)

After mechanical testing, both tibias were fixed in 10% neutral buffered formalin (NBF) and micro-CT scans of the defect and the control side were performed. The detailed procedure of the microCT analysis was already described in chapter III. For detailed information please see chapter III, part 3.2.10.

4.2.10. Histological analysis

After biomechanical testing and microCT analyses, tibial bone specimens were trimmed to 8 cm length and used for histological analysis. The detailed procedure of histological analysis was already described in chapter III. For detailed information please see chapter III, part 3.2.11.

4.2.11. Statistical analysis

Statistical analyses for the biomechanical results and for the ct-scans (microCT) were carried out using a two-tailed Mann-Whitney-U-test (SPSS 16.0, SPSS Inc.) and p-values are adjusted according to Bonferroni-Holm. Results were considered significant for p-values <0.05 .

4.3. Results

4.3.1. Cell Isolation and Differentiation

Osteoblasts (allogenic mOB and allogenic tOB) were obtained from the tibia and the mandible of sheep not included in this study. Compact bone samples were cultured in 15 ml of DMEM containing 10% FBS and 1% penicillin/streptomycin. Outgrowth of osteoblasts was observed after 5-7 days (Fig. 32). Cells were expanded to the second or third passage for following experiments.

Mesenchymal progenitor cells (allogenic MPC) were obtained from the sheep not included in this study via bone marrow aspiration from the iliac crest under general anaesthesia. Total bone marrow cells were plated at a density of $1-2 \times 10^7$ cells/cm² in complete medium and cultured until they were confluent.

Two weeks before implantation, the medium was changed in all groups to an osteogenic media to induce osteogenic differentiation. Within a few days, the cells showed a clear response to the osteogenic induction media by a pronounced morphological change. The cell morphology changed from an elongated shape to a compact cobblestone-like appearance (Fig. 33). The potential of bone marrow derived MPCs and osteoblasts (mOB and tOB) to secrete a mineralised extracellular matrix was analysed by alizarin red staining. After 2 weeks of induction, all the cells had formed extensive amounts of alizarin red-positive mineral deposits throughout the adherent layers (Fig. 34).

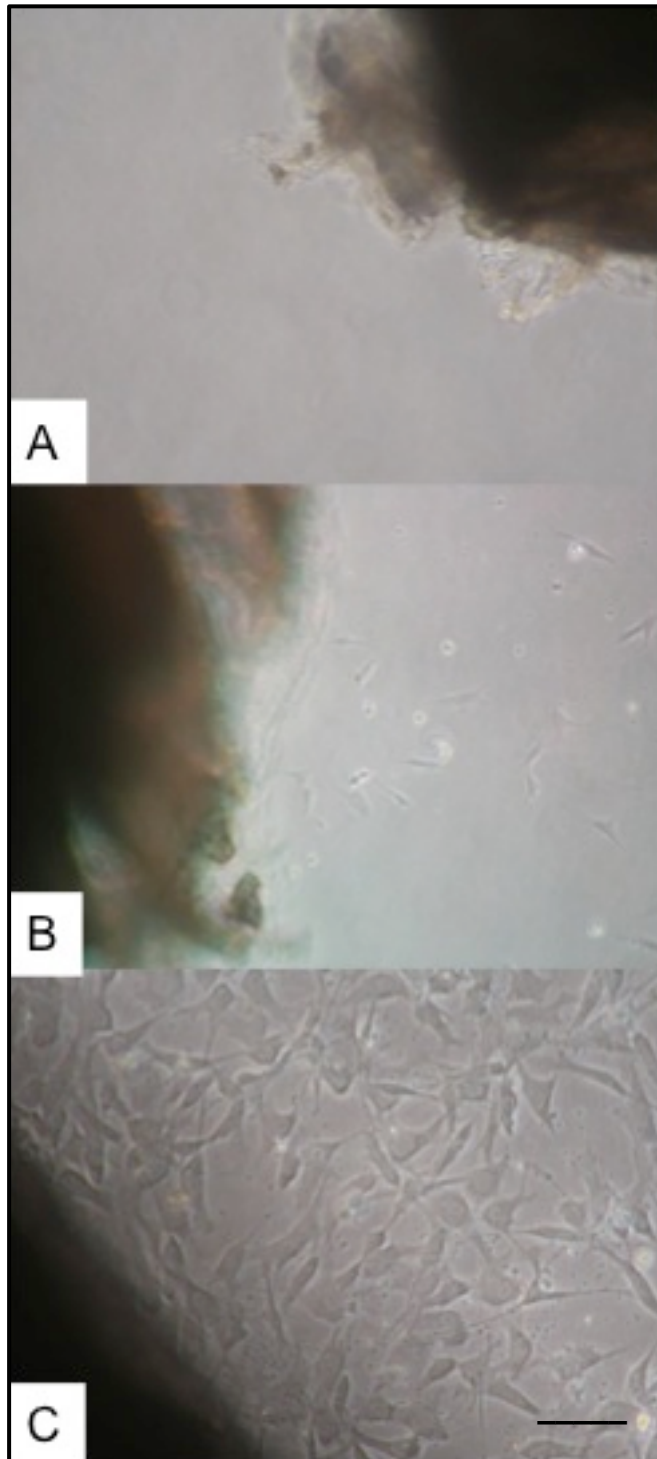


Figure 32: Culture of osteoblasts in a culture flask after 1 day (A), 7 days (B), and after 14 days (C), (scale bar = 20 μm). Outgrowth of osteoblasts was observed after 5-7 days (B).

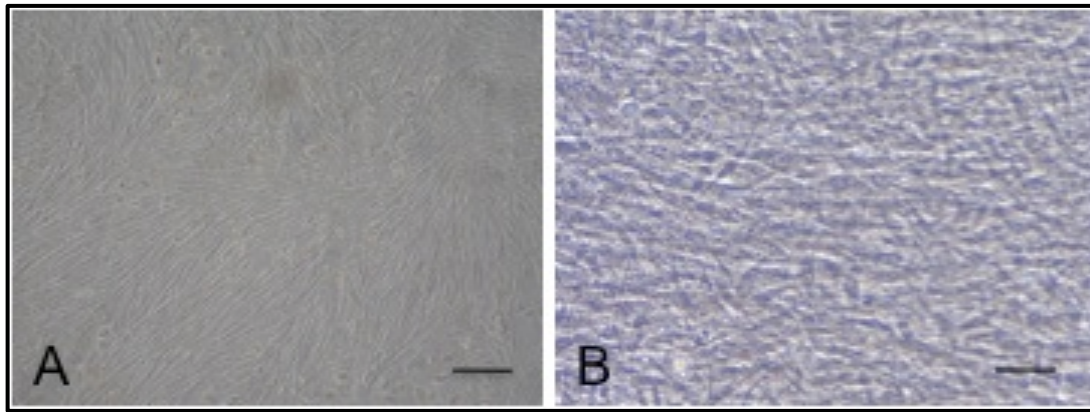


Figure 33: The cells typically have an elongated shape in regular media (A), this changed to a compact cobblestone-like appearance within days of being exposed to the osteogenic media (B), (scale bar = 100 μm).

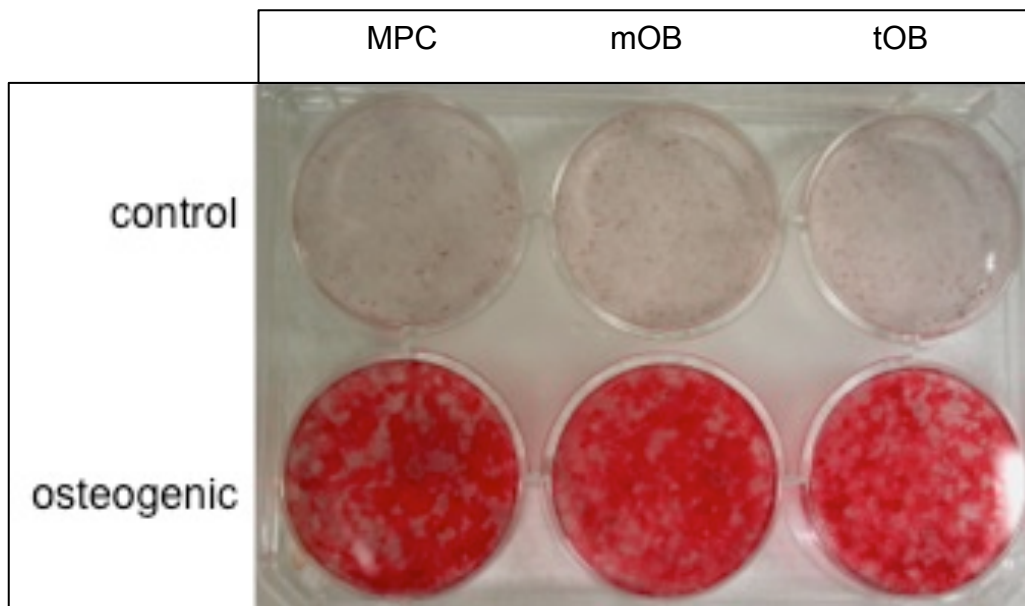


Figure 34: Alizarin red staining for MPC, mOB and tOB cultures after 14 days on 6-well plates. Under osteogenic conditions (osteogenic), all the cells secrete an alizarin-red stained mineralized matrix. The control cultures (control) stained negative.

4.3.2. Animal Surgery

In all animals, no postoperative infections or other complications were observed. The chosen 4.5 mm broad DCP was proven to be biomechanically sufficient to prevent implant failure. All animals were in good health and survived the experimental period gaining weight in the months following

surgery. In particular, the animals of the allogenic groups showed no clinical signs of implant rejection.

4.3.3. Blood analyses

Venous blood samples were taken preoperative and on days 1, 3, 7, 14, 21 after the operation from all the animals of the allogenic group and of the autologous group as a control value. The blood samples were analysed to investigate the changing white cell count (WCC) after transplantation. The blood analyses of all animals showed no signs of graft rejection.

4.3.4. Radiographic analysis

Immediately after surgery and at 3 and 6 months post-operative, conventional x-ray analyses in two standard planes (anterior-posterior and medial-lateral) were performed to assess bone formation. After surgery, the correct position of the scaffold, the plate and the screws was confirmed. After 3 months, some bone formation was observed starting from the dorsal part of the tibia where the defect is covered by the large muscle of the lower leg. The x-ray analysis after 6 months showed no loosening of the implants or movement of the scaffolds. External callus and bone formation within the defect was observed in all animals of the cell groups (Fig. 35 B-D). But no animal of the cell groups showed a complete bridging of the defect. The animals of group I (scaffold and PRP group) showed less bone formation in the defect compared to the cell groups.

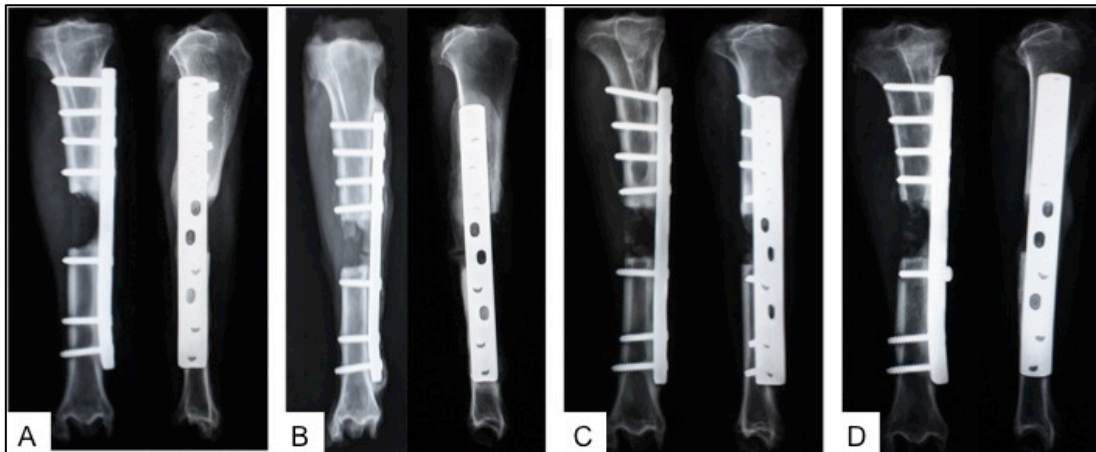


Figure 35: Representative x-ray images after 6 months of a defect reconstructed with a mPCL-TCP scaffold and PRP (A), a mPCL-TCP scaffold seeded with allogenic MPCs (B), a mPCL-TCP scaffold seeded with allogenic mOB (C) and a mPCL-TCP scaffold seeded with allogenic tOBs (D). The images show radiographic signs of bone formation within the defect in all cell groups.

4.3.5. Biomechanical testing

Biomechanical testing was performed on all specimens after 6 months. Biomechanical testing revealed an equal torsional stiffness (TS) for both osteoblast groups and the PRP group and slightly higher values for the MPC group (Fig. 36). The allogenic MPC group showed a higher torsional moment compared to the other cell groups and the PRP group (Fig. 37). For torsional stiffness and torsional moment the group with allogenic MPC showed the highest results and the PRP group the lowest results. But, no significant differences were found between all the groups at the biomechanical testing.

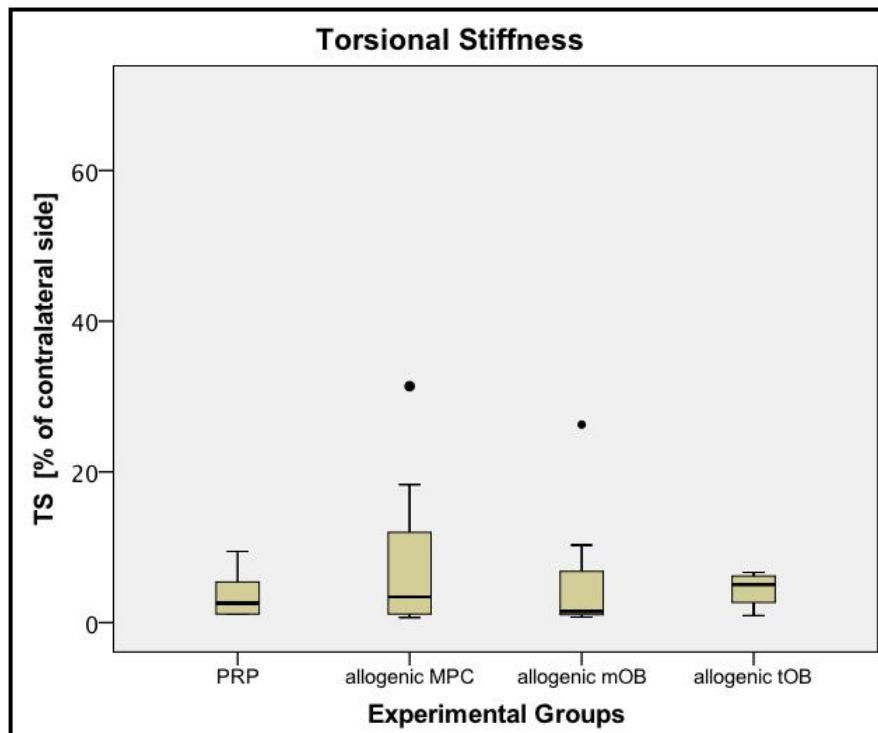


Figure 36: Results of the biomechanical testing after 6 months. Box plots demonstrating median values \pm 1st and 3rd quartile of torsional stiffness in all experimental groups. Error bars represent maximum and minimum values.

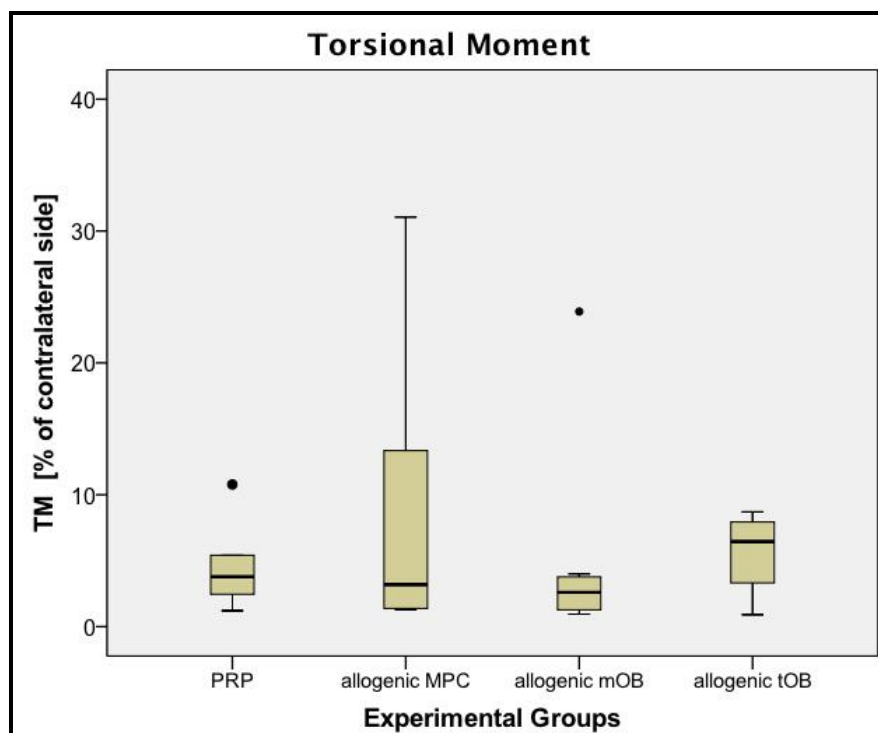


Figure 37: Results of the biomechanical testing after 6 months. Box plots demonstrating median values \pm 1st and 3rd quartile of torsional moment of the experimental groups. Error bars represent maximum and minimum values.

4.3.6. Computed tomography (microCT)

MicroCT analysis confirmed the trend of the results from the conventional x-ray analysis regarding union rates and the amount of new bone formation. In the 3D reconstructions, all cell groups showed small amounts of new bone formation in the defect, mainly at the opposite site of the plate (Fig. 38). The mean values of newly formed bone in the MPC group was higher compared to the osteoblast groups and the PRP group (Fig. 39). The two osteoblast groups showed the lowest median values of newly formed bone. In all samples, newly formed bone was still less compared to the amounts determined for the same anatomic level of the contralateral hind limbs.

The new bone was distributed throughout the external, scaffold and endosteal area of the defect with the highest amount of newly formed bone in the external area (Fig. 40.).

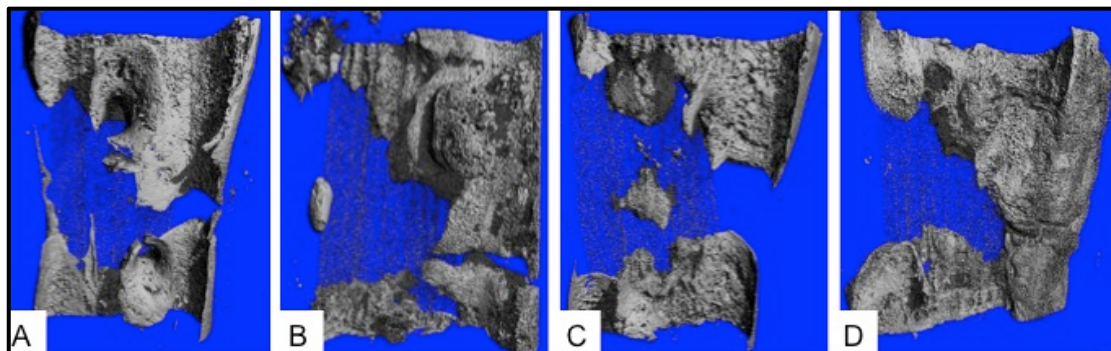


Figure 38: Representative 3D reconstructions of microCT scans of 3 cm tibial defects 6 months after surgery of the PRP-Group (A), the allogenic MPC group (B), the allogenic mOB group (C) and the allogenic tOB group (D).

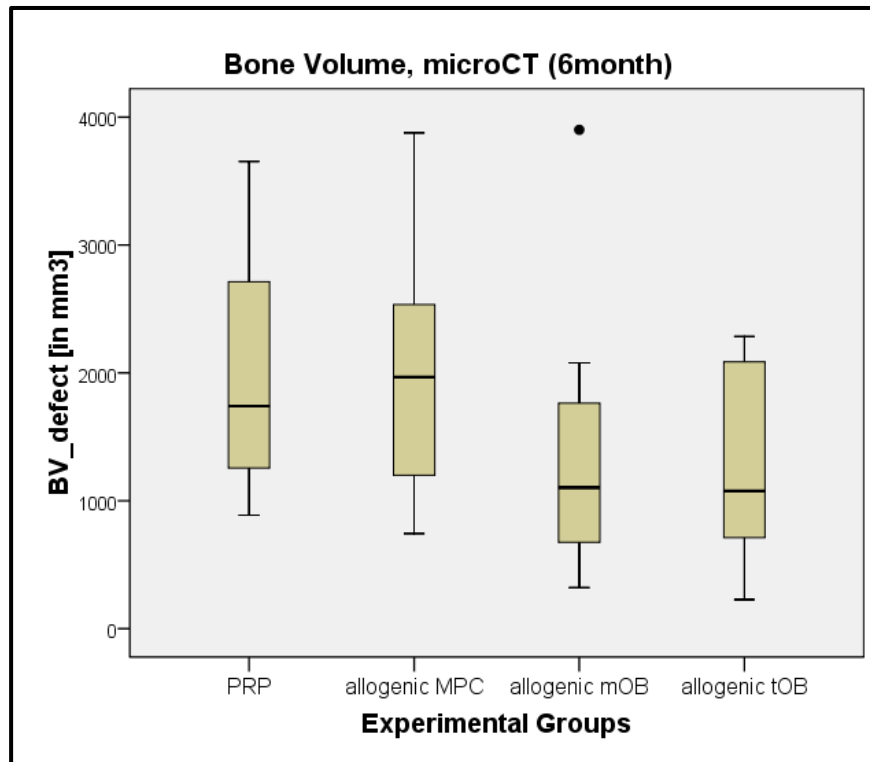


Figure 39: Results of the microCT scanning after 6 months. Box plot demonstrating median amounts of newly formed bone \pm 1st and 3rd quartile within the 3 cm defects 6 months after surgery. Error bars represent maximum and minimum values.

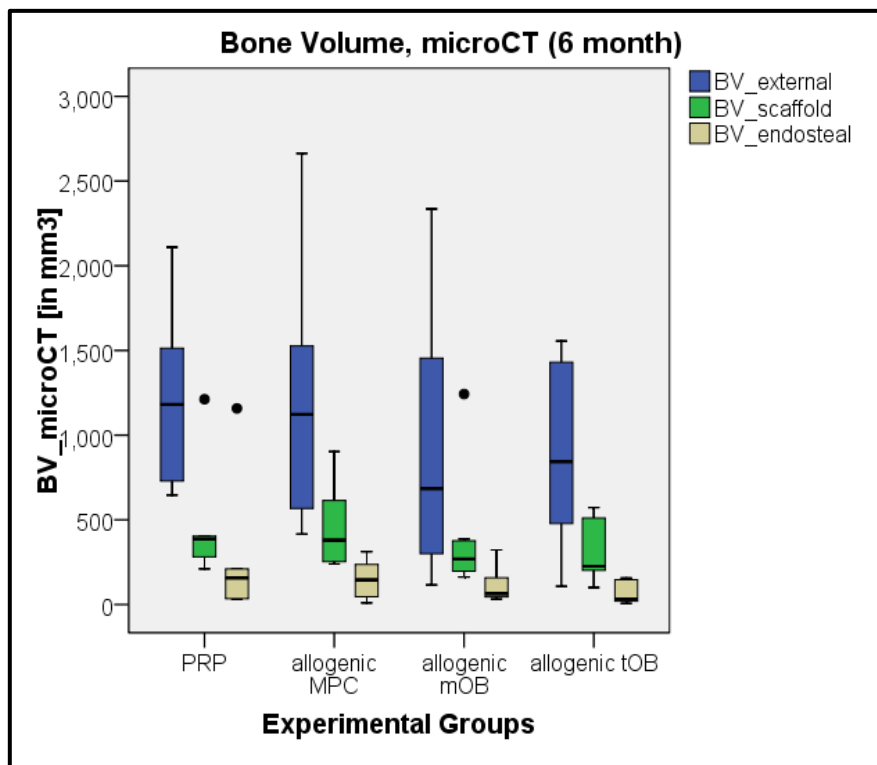


Figure 40: Results of the microCT scanning after 6 months. The amount of bone volume within the defect was distributed to the area outside the scaffold (external), the area within the scaffold (scaffold) and the inner part of the scaffold (endosteal). Box plots are demonstrating median amounts of newly formed bone \pm 1st and 3rd quartile 6 months after surgery of the operated site. Error bars represent maximum and minimum values.

4.3.7. Histology/Immunohistochemistry

The macroscopic overview of the implanted scaffolds before processing for histology showed good integration of the scaffold with the host bone in all animals of all experimental groups. The mPCL-TCP scaffold was still in place and had not been resorbed. Histological examinations of decalcified samples were performed after 12 weeks. Representative H&E staining of all experimental groups demonstrated a good integration of the scaffold to the host bone on the proximal as well as the distal side (Fig. 41 A, C, E, G). Notably, new bone formation was seen in many pores within the mPCL-TCP

scaffolds in all cell groups as well as the PRP group (the scaffold itself is revealed in the histology slices as empty circular “holes” and “bars” where the scaffold struts used to reside, due to the dissolution of the mPCL implant by xylene during processing) (Fig. 41 B, D, F, H).

These results confirm our microCT evaluation and demonstrate a limited amount of bone tissue growing into the whole scaffold area in all the cell groups as well as the PRP group.

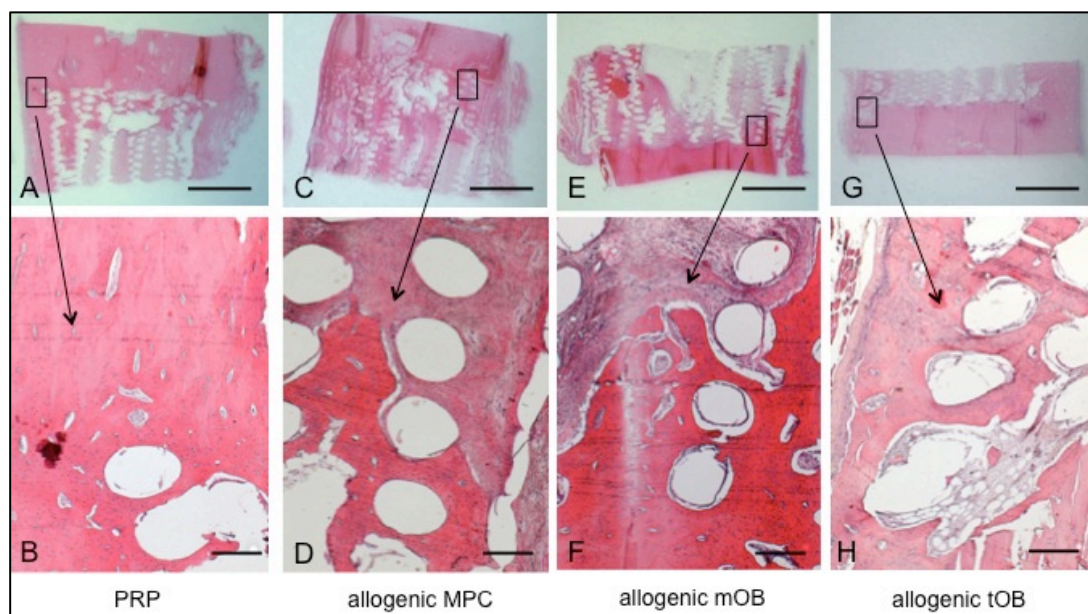


Figure 41: Representative H&E staining of all experimental groups after 6 months. The proximal parts of the defect showed a good integration of the scaffold as well as a good bonding of the regenerated bone to the host bone (A, C, E, G; bar=0.5cm). The solvents used during the preparation of the histological sections resulted in the mPCL–TCP scaffold material being dissolved during embedding. Hence mPCL–TCP struts are represented in histological sections as empty “holes” of similar geometry. All experimental groups showed new bone formation around the scaffold struts (B, D, F, H; bar =100 μ m).

4.4. Discussion

Bone regeneration and fracture healing in the field of orofacial surgery is indistinguishable to the axial skeleton. On both injury sites, the regeneration

process goes through the same stages, however several *in vitro* and *in vivo* experiments showed differences in the repair mechanism of these cells. Leucht et al. showed that cells isolated from orofacial bones respond to local cues, adopt their Hox status of their new locale, and robustly contribute to the formation of bony regeneration²⁹⁵. In contrast, cells isolated from the axial skeleton maintain their Hox status when transplanted to an orofacial bone defect with a resulting disruption in bone regeneration. Furthermore, Akintoye et al. found in their experiments the existence of skeletal site properties of orofacial cells and axial cells based on their different embryological origins²⁹⁶.

Cells used in our experiments were isolated from orofacial bones (mOB), from axial bones (tOB) and from the iliac crest (MPC). While finding clear differences in the osteogenic potential of osteoblasts and mesenchymal progenitor cells in further *in vitro* and *in vivo* experiments, we were not able to detect any differences in their bone regenerative potential in a critical sized tibial bone defect in a large animal model.

The use of allogenic cells for clinical therapy is already established and in routine use in other areas of medicine and shows several advantages compared to the use of autologous cells^{268,269}. In further *in vitro* experiments by our group, we found a similar expression of surface markers between mesenchymal stem cells and osteoblasts. Both cell populations were negative for the haematopoietic surface markers CD 45 and CD 31 and positive for CD 29 and CD 44²⁸³.

Hence, a non-autologous use of all cell types was performed to have all the positive effects of allogenic cell transplantation. The results of our study demonstrate, that allogenic cell transplantation of mesenchymal progenitor cells and differentiated cells (mOB and tOB) is a safe method without showing any signs of immunological reaction.

We were able to demonstrate clear advantages in the use of differentiated cells for bone regeneration in a small animal model. The results of the small animal model study could not be confirmed in our large animal study. We were unable to see any differences in the regenerative potential of mesenchymal progenitor cells compared to the application of differentiated cells (osteoblast; mB and tOB) regarding the bone volume within the defect or the biomechanical properties. All the cell based tissue engineering approaches in our study showed similar results compared to the cell-free approaches (Scaffold and PRP). No beneficial effect of the cells could be demonstrated.

This demonstrates once again the importance of standardised large animal models with validated experimental protocols to compare outcomes of each different bone engineering approach. Results gained from *in vitro* experiments or small animal models can't be transferred to the clinical setting. New tissue engineering approaches need to be tested in large animal models to generate advanced information about the procedures and to make the decision to move to a clinical study.

4.5. Conclusion

The present study showed that the use of allogenic differentiated cells in orthopaedics for bone regeneration is a safe method for a cell based tissue-engineering approach. We detected no adverse immunological response. But, we were not able to show a positive effect of the cells on bone regeneration in a large animal model compared to the cell-free approach. Therefore, the cell-based tissue engineering approaches for large bone defect regeneration still needs further optimisation before translating these novel procedures into clinical trials.

Chapter V – New concepts in bone tissue engineering - Delayed Injection of allogenic mesenchymal progenitor cells for the reconstruction of large bone defects

5.1. Introduction

Cell based tissue engineering approaches are common concepts for bone regeneration in the area of regenerative medicine. Several experts demonstrate promising results *in vitro* as well as in small animal models.

Autologous and allogenic mesenchymal progenitor cells (MPCs) have been injected into tissue sites or infused into the blood stream and have been observed to congregate to tissue sites of injury involving broken or inflamed blood vessels such as an acute myocardial infarct, stroke, spinal cord injury, lung- and pulmonary disease, burns and wound healing. Experiments from different groups indicate that BMSCs secrete numerous cytokines and chemokines well known to be important to stimulate and achieve wound healing, such as vascular endothelial growth factor alpha (VEGF alpha), epidermal growth factor-alpha (EGF alpha), insulin growth factor-1 (IGF1), keratinocyte growth factor (KGF), angiopoietin-1 (Ang1), stromal derived factor-1 (SDF1), macrophage inflammatory protein-1 alpha and beta (MIP1

alpha and beta), and erythropoietin in greater amounts than dermal fibroblasts²⁹⁷.

Caplan et al. recently reported, that MPCs are clinically active at different tissue sites, that MPCs are pericytes and can be isolated from any vascularized tissue, and that MPCs secrete large quantities of a variety of bioactive molecules as part of their local trophic and immunomodulatory activities²⁷¹.

Despite successful results in clinical trials in other areas of medicine, the translation of cell based tissue engineering concepts into clinical practice is still experimental in the area of trauma and orthopaedic surgery. The functional role of MPCs in the regeneration of different tissues is not fully understood and often discussed controversially. Kon et al. reported, that the regenerative effects of mesenchymal stem cells are due to their structural contribution to tissue repair and to their immunomodulatory and anti-inflammatory activity, through direct cell-cell interaction or secretion of various factors²⁹⁸.

In the histological analyses of our samples in previous experiments we found most of the transplanted cells to be necrotic. This might be due to the initial inflammatory phase, due to low nutrition supply and local hypoxia postoperative.

Based on our results, we hypothesised that the technique for cell delivery needed to be modified. Therefore, we modified the experimental setup of the cell delivery procedure. We decided to implant the scaffold without cells, and

postponed the cell delivery until the inflammatory phase had passed (3-4 weeks after scaffold implantation). Afterwards, the cell suspension was injected percutaneously into the prior implanted scaffold.

We hypothesized that the technique of delayed cell injection of allogenic stem cells would lead to a higher cell survival rate and therefore to increased bone formation, which would be comparable to the application of autologous bone grafts (ABG).

The aim of this study was to assess and compare the regenerative potential of allogenic bone marrow mesenchymal progenitor cells (allogenic MPC's) in combination with a mPCL scaffold with the application of autologous bone grafts (ABG) in a critically sized segmental bone defect in sheep tibiae.

5.2. Material and Method

5.2.1. Scaffold fabrication and preparation

Bioresorbable cylindrical scaffolds of medical grade poly-caprolactone (mPCL), (outer diameter: 16 mm, height: 30 mm, inner diameter: 8 mm) were used in this study. The scaffolds are produced by fused deposition modelling (FDM). The scaffolds have a porosity of 70% and a 0/90° lay down pattern (Fig. 42). This architectural layout was particularly suitable for load bearing tissue engineering applications since the fully interconnected network of scaffold fibres can withstand early physiological and mechanical stress in a manner similar to cancellous bone. Moreover, the architectural pattern allowed the retainment of coagulating blood during the early phase of healing, and bone ingrowth at later stages. Prior to surgery, all scaffolds were

surface treated for six hours with 1M NaOH and washed five times with PBS to render the scaffold more hydrophilic. Scaffold sterilization was achieved by incubation in 70% ethanol for 5 min and UV irradiation for 30 min.

To enhance osteoinduction, mPCL scaffolds were coated with a layer of calcium phosphate (CaP). The coating process consisted of three steps: surface activation with alkaline treatment (Sodium hydroxide (NaOH)); treatment with simulated Body Fluid 10x (SBF10x) to deposit the CaP; and post-treatment with NaOH. For 1 L of solution, reagents were dissolved in ddH₂O in the following order: 58.430g NaCl, 0.373g KCl, 3.675g CaCl₂·2H₂O and 1.016g MgCl₂·6H₂O. The next reagent (1.420g of Na₂HPO₄) was dissolved separately in 20 mL of ddH₂O and added drop by drop into the main solution while maintaining the pH level at 4 by adding Hydrochloric acid (HCl) 32% in order to avoid precipitation of calcium cations and phosphate anions. The tubes were first cleaned by immersion in 70% ethanol solution under vacuum for 15 min for the purpose of removing entrapped air bubbles, then the structures were immersed into pre-heated (37 °C) NaOH 2M and a 5 min vacuum treatment was performed at room temperature. For the rest of the activation steps, the scaffolds were placed at 37 °C for 30 min to accelerate the etching process. The scaffolds were then rinsed with ddH₂O until the pH level dropped to approximately 7. Meanwhile, NaHCO₃ was added to the SBF10x solution until a pH of 6 was reached. This activated SBF solution was filtered (0.2 µm filter) and another 5 min vacuum treatment at room temperature was performed to ensure that the solution fully penetrated the tubes. The samples were thereafter placed at 37 °C for another 30 min. The solution was replaced with freshly activated and filtered

SBF and placed again at 37 °C for 30 min. The tubes were rinsed in ddH₂O and then immersed in pre-heated NaOH 0.5 M for 30 min at 37 °C. Finally the tubes were rinsed with ddH₂O and the pH level was adjusted to approximately 7 and then dried overnight in a dessicator.

Prior to cell loading, the scaffolds were modified by making 3 large punch holes on the back side of the scaffold (diameter 4mm) and 4 smaller holes on the front side (diameter 3mm) (Fig. 42). The holes on the back were placed directly over the neuro-vascular bundle in the dorsal part of the bone defect, to allow the ingrowth of new blood vessels (Fig. 43 C/D). The 4 holes in the front part were used for the delayed cell injection into the scaffold after 4 weeks and were placed next to the plate (Fig. 43 A/B).

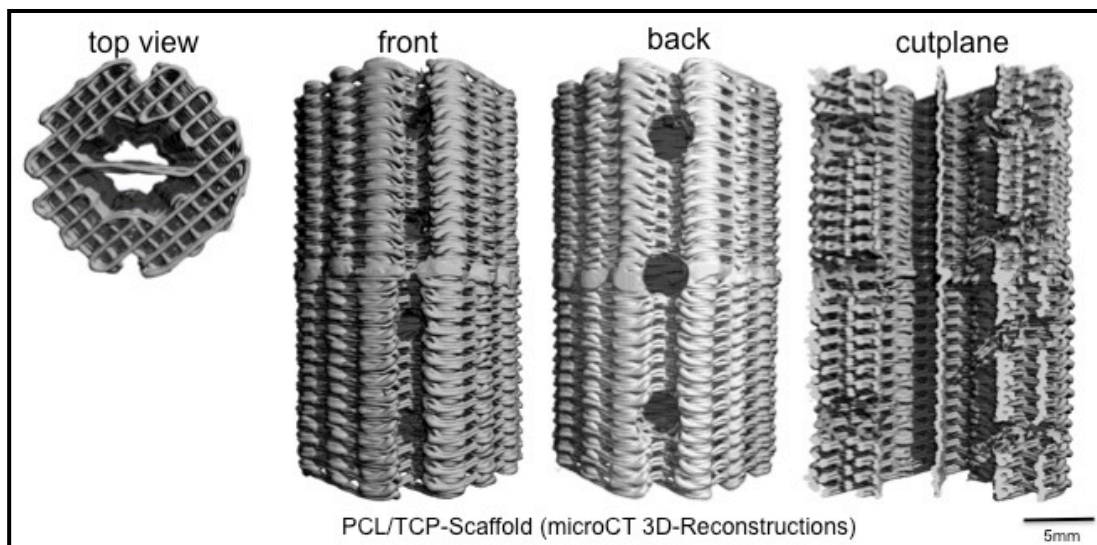


Figure 42: The new scaffold design of the mPCL-CaP scaffolds. The 4 holes on the front side were used for the delayed cell injection, whereas the 3 holes on the back side should support the ingrowth of new blood vessels into the scaffold.

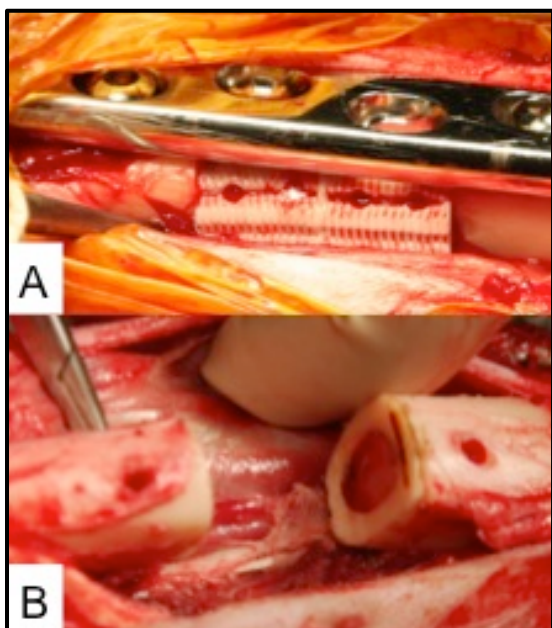


Figure 43: The 4 holes on the front side were used for the delayed cell injection (A) and were placed next to the plate, where as the 3 holes on the back side should support the ingrowth of new blood vessels into the scaffold (B).

5.2.2. Animal study

Sixteen male sheep (40-50 kilogram, age 7-8 years) were used in this project (Table 7). An Animal Ethics Approval Certificate had been obtained. Animal surgery was performed at the QUT Medical Engineering Research Facility, The Prince Charles Hospital, Chermside. Owing to ethical reasons to lower the number of animals, the results of the experimental groups will additionally be compared to the autologous bone graft group (ABG), (positive control group) from the work of Dr. Johannes Reichert, who included these experiments in his thesis. There were 8 animals in each group and all were euthanized after 12 months.

The research plan for this study is structured as follows:	Group I	Group II
	mPCL-CaP + allogenic MPC (100x10⁶Cells)	ABG
Total	8	8

Table 7: The research plan with details of the animal treatment groups. The time duration of this experiment was 12 months.

5.2.3. Animal Surgery

The anaesthetic procedures, the pre-operative treatment and the surgical procedures were described in chapter III. For detailed information please see chapter III, part 3.2.4.

5.2.4. Experimental Groups

- Group I

All the scaffolds (mPCL-CaP) in this group were implanted into the defect site and the wound closed in layers. Delayed cell injection was performed four weeks after implantation of the scaffolds (see part 5.2.5. cell delivery). Prior to injection, the allogenic ovine mesenchymal progenitor cells were obtained from Merino sheep that were not included in this study (allogenic cells). Bone marrow aspirates were obtained from the iliac crest under general anaesthesia. Total bone marrow cells ($5-15 \times 10^6$ cells/ml) were plated at a density of $10-20 \times 10^6$ cells/cm² in complete medium consisting of low glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were subsequently plated at a density of 10^3 cells/cm². Two weeks before injection, the medium was changed to an

osteogenic media (DMEM, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 10 µl/ml β-glycerophosphate, 1 µl/ml ascorbic acid and 1 µl/ml dexamethasone) to induce osteogenic differentiation.

- Group II

Autologous cancellous bone graft (ABG) was harvested from the left iliac crest. The surgical area was shaved and disinfected with 0.5% chlorhexidine red in 70% ethanol. A 5 cm incision was made following the iliac crest, the inserting musculature was carefully detached and the cortical bone of the lateral os ileum was fenestrated (2 x 2 cm) using a hammer and osteotome. Care was taken not to fracture the *ala ossis ilii*. The resulting lid was carefully removed with a raspator and the cancellous bone harvested utilizing a bone curette. Then, the lid was reinserted, the musculature was reattached with 2-0 Vicryl sutures (Ethicon), and the wound closed in layers. The closed wound was sprayed with Opsite (Smith and Nephew).

5.2.5. Cell delivery

Four weeks after implantation of the scaffolds, the allogenic MPCs were injected percutaneously into the scaffold. For this procedure, 100×10^6 cells were detached from the cell culture flasks using a cell scraper so not to destroy the extracellular matrix. The resulting cell sheets were transferred to large petri dishes filled with standard media (Fig. 44 A). The cell sheets were dissected using a sharp scalpel blade to get a smooth solution of the cells (Fig. 44 B). Finally the cell solution (4 ml) was equally transferred to four 5ml syringes under sterile conditions (Fig. 44 C-E). Cell injection to the bone

defects was performed under general anaesthesia and sterile conditions in the operating room.

The right hind limb was carefully shaved and thoroughly disinfected with 0.5% chlorhexidine solution red in 70 % ethanol. The animal torso and surroundings were covered with sterile sheets. The DCP plate was localized through the skin and another plate was placed from outside to identify the exact position of the plate holes (Fig. 45 A). A sharp needle (14 gauge) was placed through the proximal hole of the scaffold. After that, three other needles were placed in the same way into the other holes (Fig. 45 B).

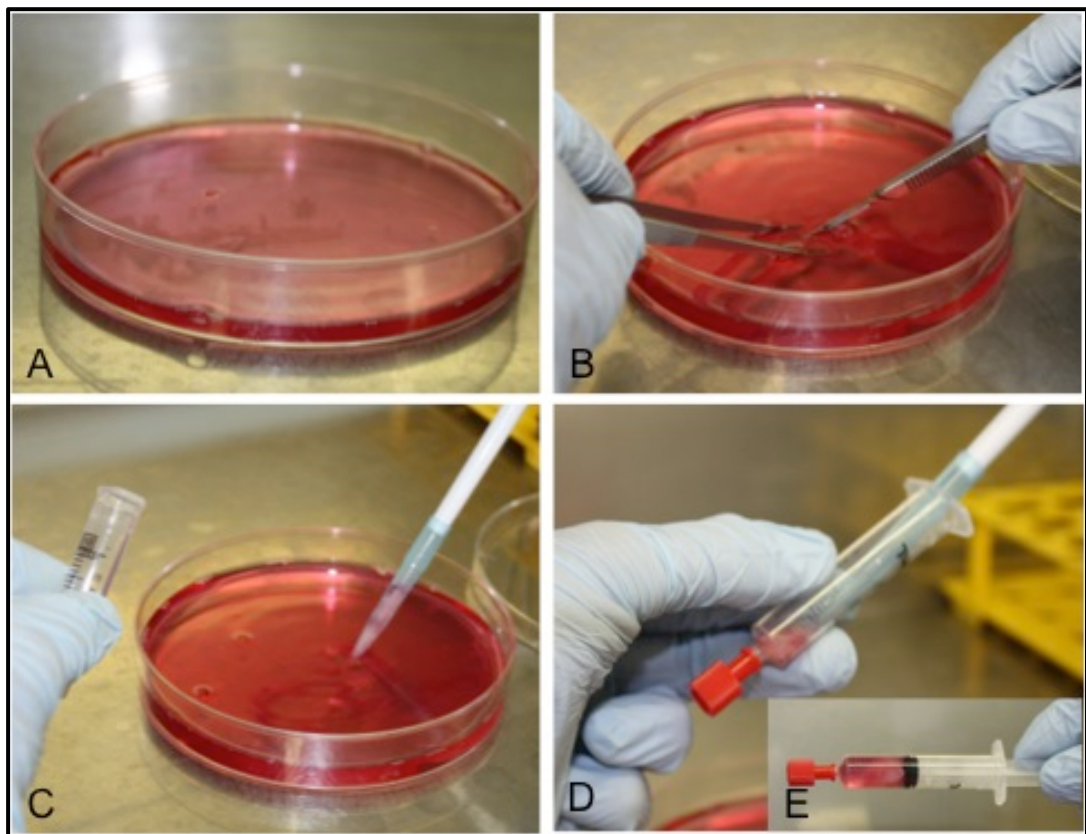


Figure 44: Cells for the injection were detached using a cell scraper and put into large petri dishes with standard media (A). The cell sheets were dissected using a sharp scalpel blade (B) and the transferred to four 5ml syringe under sterile conditions (D/E).

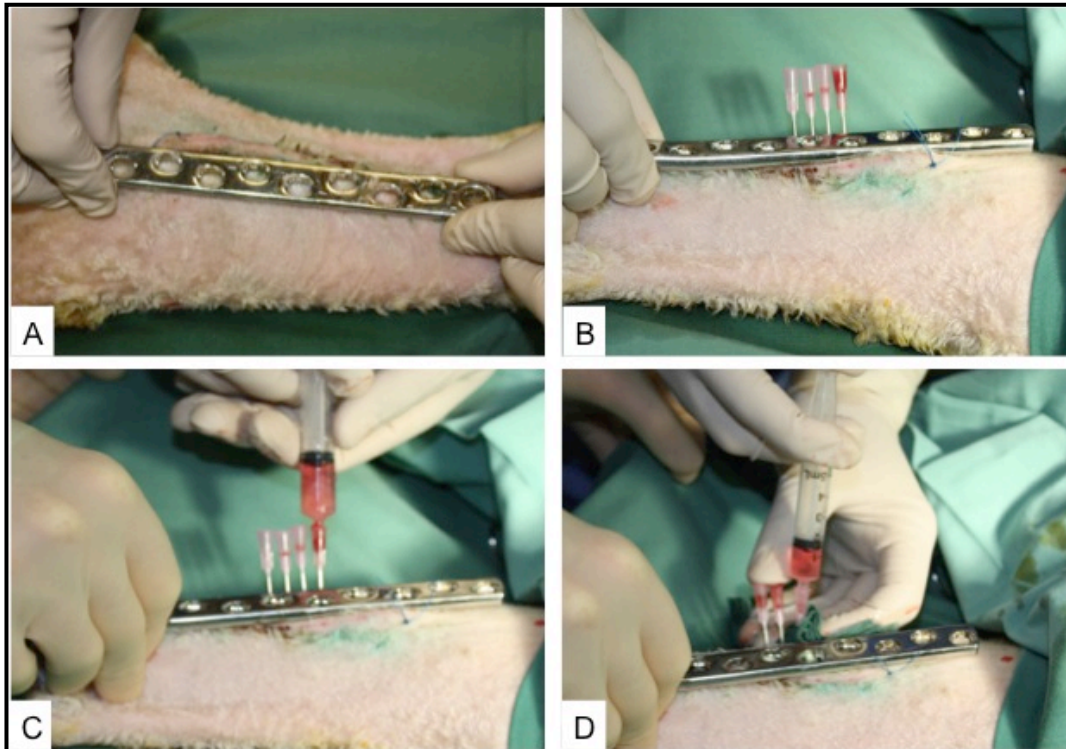


Figure 45: The plates were localized through the skin and another plate was placed from outside to identify the exact position of the plate holes (A). Four needles were placed into the holes of the scaffold (B), and the cell suspensions were injected into the defect (C/D).

The cell suspension (1 ml) from every prepared syringe was injected into the defect and the previously implanted scaffold starting at the proximal end of the defect (Fig. 45 C/D; Fig. 46). The wound was sprayed with Opsite (Smith and Nephew), covered with pads and bandaged (Vetrap, 3M). After recovery from anaesthesia, animals were allowed unrestricted weight bearing.

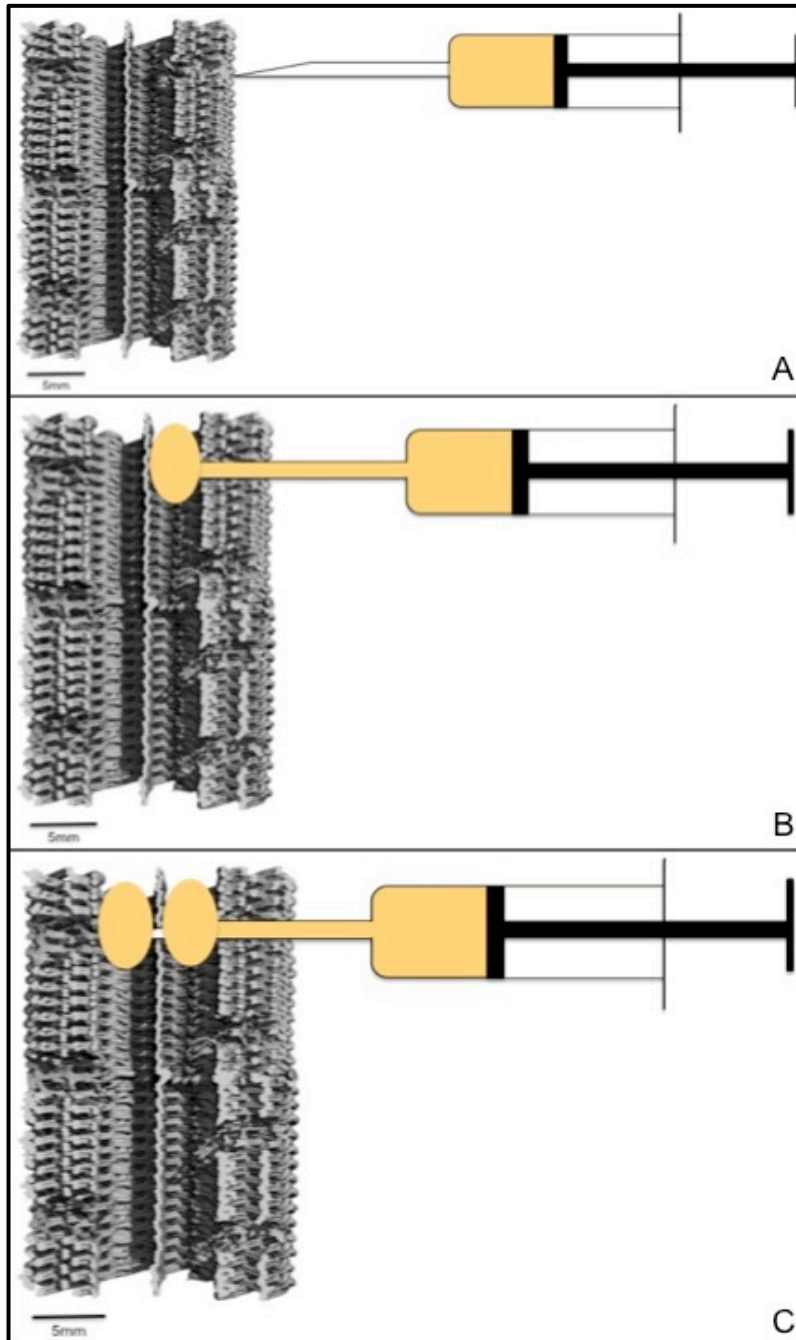


Figure 46: Schematic description of the cell injection process. The syringe was placed in the hole of the scaffold and half of the cell solution was injected into the anterior part of the scaffold (A/B). The needle was then put through the middle wall of the scaffold and the rest of the cell solution was injected into the posterior part of the scaffold (C).

5.2.6. Radiography analysis

Throughout the study, x-rays were taken after 3, 6 and 12 months, to determine the time of bridging of the defect in the different experimental groups. Conventional x-ray analysis (3.2 mAs; 65kV) was performed in two standard planes (anterior-posterior and medial-lateral). At euthanasia, the gross morphology and mobility at host-graft junctions was clinically assessed and the findings carefully documented and photographed.

5.2.7. Biomechanical evaluation

To determine the integration of the scaffolds into the bone and the recovery of the biomechanical function of the affected tibias, biomechanical testing was performed. The detailed procedure of biomechanical testing was described in chapter III. For detailed information please see chapter III, part 3.2.9.

5.2.8. Computed tomography (microCT)

After mechanical testing, both tibias were fixed in 10% neutral buffered formalin (NBF) and micro-CT scans of the defect and the control site were performed. The detailed procedure of the microCT analysis was described in chapter III. For detailed information please see chapter III, part 3.2.10. The microCT analyses of the specimens in this study are still in progress.

5.2.9. Histological analysis

After biomechanical testing and microCT analyses, tibial bone specimens were trimmed to 8 cm length and used for histological analysis. The detailed procedure for histological analysis was described in chapter III. For detailed information please see chapter III, part 3.2.11. Due to the associated time frame needed for histological analyses of large bone specimens (6 – 12 months, decalcification and embedding) the histological analyses of the specimens from this study are still in progress.

5.2.10. Statistical analysis

Statistical analyses for the biomechanical results and for the ct-scans (microCT) were carried out using a two-tailed Mann-Whitney-U-test (SPSS 16.0, SPSS Inc.) and p-values are adjusted according to Bonferroni-Holm. Results were considered significant for p-values <0.05 .

5.3. Results

5.3.1. Cell Isolation and Differentiation

Mesenchymal progenitor cells (allogenic MPC) were obtained from Merino sheep not included in this study via bone marrow aspiration from the iliac crest under general anaesthesia. Total bone marrow cells were plated at a density of $1-2 \times 10^7$ cells/cm² in complete medium and cultured until they were confluent.

Two weeks before implantation, the medium was changed in all groups to an osteogenic media to induce osteogenic differentiation. Within a few days, the cells showed a clear response to the osteogenic induction media by a pronounced morphological. The cell morphology changed from an elongated shape to a compact cobblestone-like appearance. The potential of bone marrow derived MPCs to secrete a mineralised extracellular matrix was analysed by alizarin red staining. After 2 weeks of induction, all the cells had formed extensive amounts of alizarin red-positive mineral deposits throughout the adherent layers (see Fig. 33/34, chapter 4).

5.3.2. Animal Surgery

In all animals, no postoperative infections or other complications were observed. The chosen 4.5 mm broad DCP was proven to be biomechanically sufficient to prevent implant failure. After 12 months, bone overgrowth was observed on nearly all plates. All animals were in good health and survived the experimental period, gaining weight in the months following surgery. In

particular, the animals of the allogenic group showed no clinical signs of implant rejection.

5.3.3. Radiographic analysis

Immediately after surgery and after 3, 6 and 12 months, conventional x-ray analyses in two standard planes (anterior-posterior and medial-lateral) were performed to assess bone formation. After surgery, the correct position of the scaffold, the plate and the screws were confirmed. After 3 months, bone formation was observed in the ABG group and the cell group starting from the dorsal part of the tibia where the defect is covered by the large muscle of the lower leg. The x-ray analysis after 6 months showed no loosening of the implants or movement of the scaffolds. Increasing bone formation was observed in the ABG group and the cell group. After 12 months, no implant loosening was observed in any animals. Complete bridging of the defects in the ABG group and the cell group was observed in all animals (Fig. 47 B/C).



Figure 47: Representative x-ray images after 12 month of a defect reconstructed with a mPCL-CaP scaffold and allogenic MPCs (delayed injection) (A), and a defect reconstructed with the application of autologous bone grafts (ABG) (B). The images show radiographic signs of bone formation within the defect in the cell group as well as the ABG group.

5.3.4. Biomechanical testing

Biomechanical testing was performed on all specimens after 12 months. Biomechanical testing revealed a higher torsional stiffness (TS) and a higher torsional moment (TM) for the cell group compared to the scaffold only group. The results were not statistically significant. The ABG group showed the highest results for both, torsional moment and torsional stiffness, with significantly higher results compared to the scaffold only group and no significantly higher results compared to the cell group (Fig. 48/49).

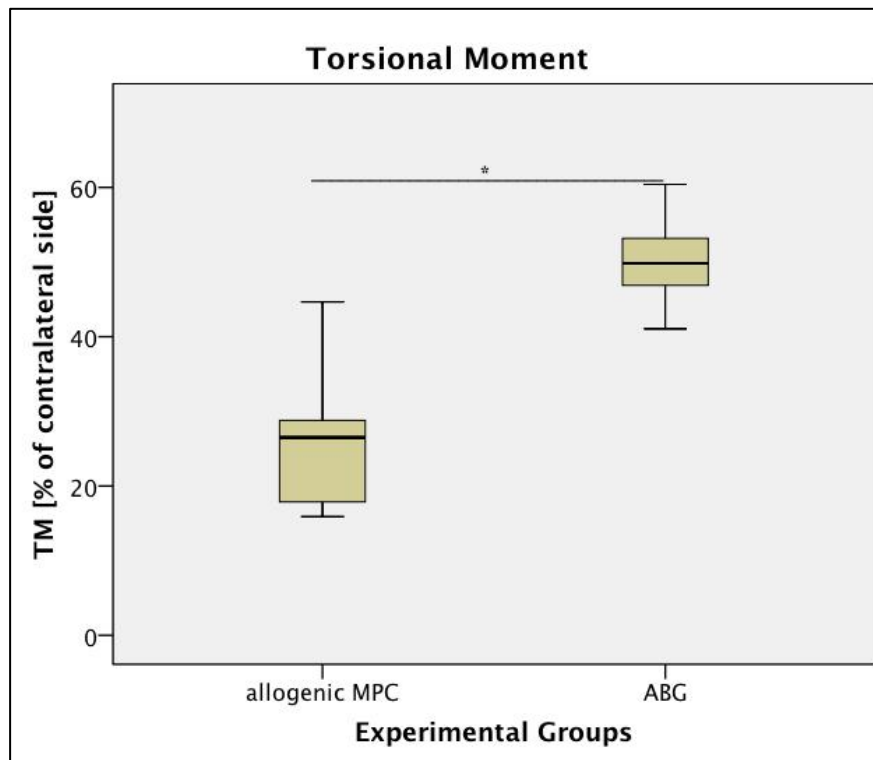


Figure 48: Biomechanical analyses (torsional moment) after 12 months. Box plots demonstrating median values \pm 1st and 3rd quartile of torsional stiffness in all experimental groups. Error bars represent maximum and minimum values. Asterisks indicate statistical significance ($p < 0.05$).

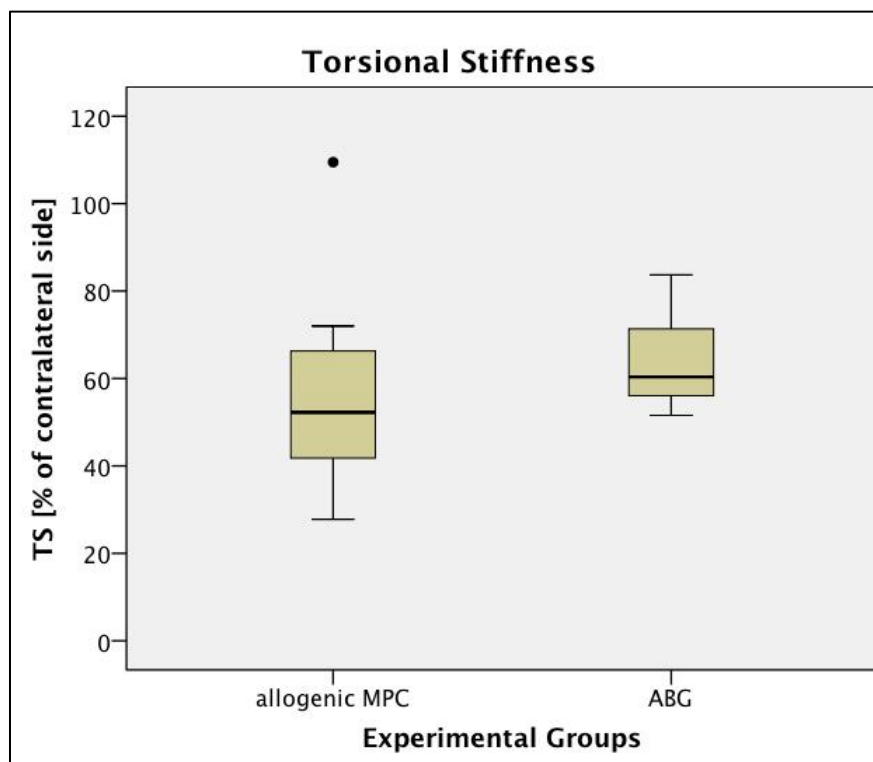


Figure 49: Biomechanical analyses (torsional stiffness) after 12 months. Box plots demonstrating median values \pm 1st and 3rd quartile of torsional stiffness in all experimental groups. Error bars represent maximum and minimum values. Asterisks indicate statistical significance ($p < 0.05$).

5.3.5. Computed tomography (microCT)

MicroCT analysis confirmed the trend of the results from the conventional x-ray analysis regarding union rates and the amount of new bone formation.

In the 3D reconstructions, the groups showed large amounts of new bone formation in the defect with a complete bridging of the defect (Fig. 50). The mean values of newly formed bone in the MPC group was slightly higher compared to the ABG group without significant differences ($p=0.284$) (Fig. 51).

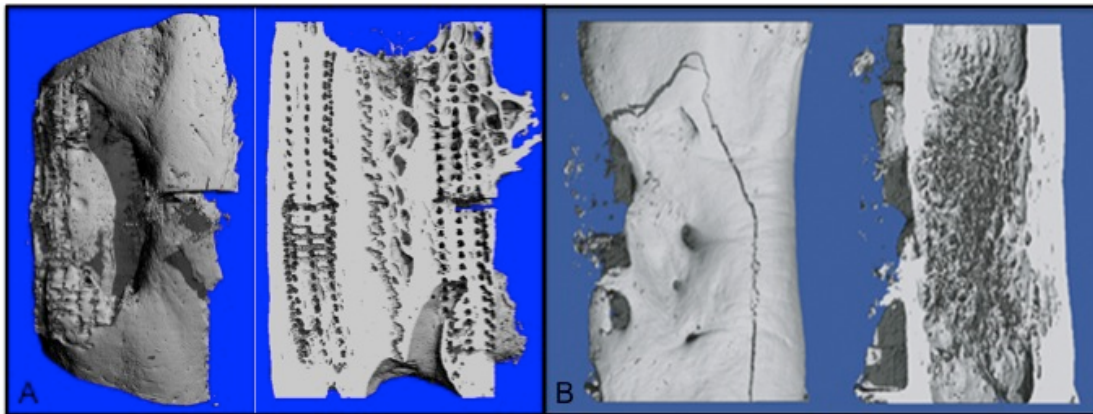


Figure 50: Representative 3D reconstructions of microCT scans of 3 cm tibial defects 12 months after surgery of the allogenetic MPC group (A) and the ABG group (B).

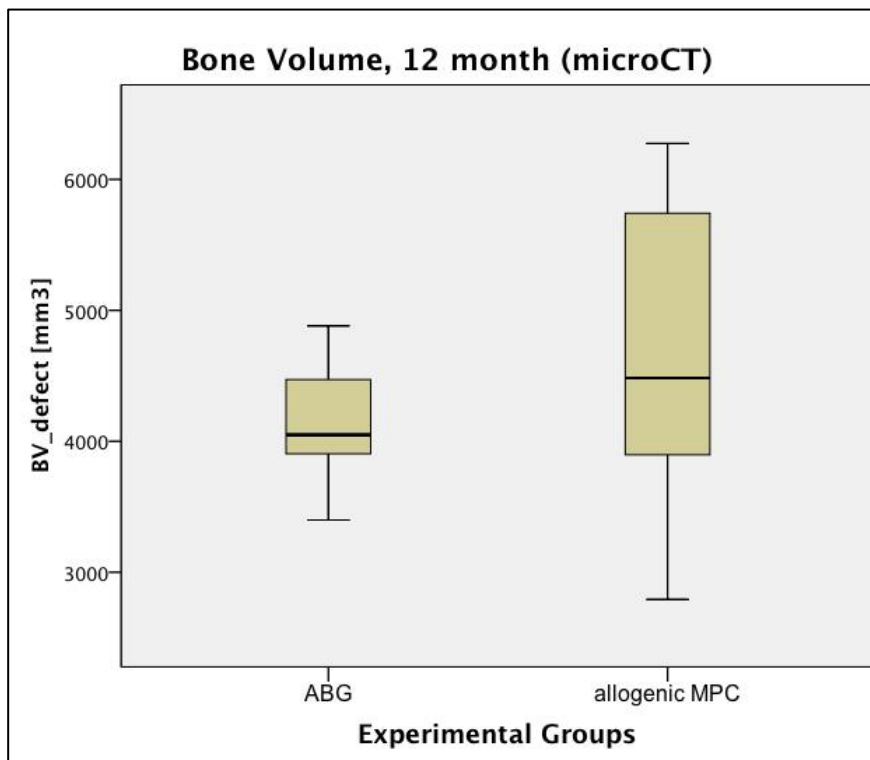


Figure 51: Results of the microCT scanning after 12 months. Box plot demonstrating median amounts of newly formed bone \pm 1st and 3rd quartile within the 3 cm defects 12 months after surgery. Error bars represent maximum and minimum values.

5.4. Discussion

Cell-based therapies are promising strategies in regenerative medicine. The future of these cell-based strategies will be influenced by the cell source used, either allogenic or autologous cells and the donor site of the cells. Mason and Dunhill assessed the advantages and disadvantages of autologous and allogenic human cells for regenerative medicine⁷⁹. There are a number of issues that need to be considered comparing the efficiency and efficacy when comparing allogeneic and autologous cells (Table 8/9) and more specifically for scaffold-cell based bone engineering.

The treatment of large segmental bone defects requires a large number of cells with a high osteogenic differentiation potential. If using an autologous cell source, the cost and the time to get the required cells is high. Therefore the use of an allogeneic cell source which can be used as an “off-the shelf product” is preferable to use a high number of cells for the treatment of bone defects within an acceptable time frame, if using these concepts in traumatic bone defects. The major disadvantage of using allogeneic cell sources is the possible risk of an immune reaction (Table 8/9).

Advantages

• Allogeneic

- Producing cells for many patients is more efficient
 - Scale-up can go much further
 - Quality control (QC) can be applied to larger lots
 - Existing attachment cell technology for production scale is useful
 - Material of high consistency
 - Allows high patient throughput
- Cells are always available
 - Can address emergency indications
 - Represents a good commercial opportunity for cell suppliers/contract manufacturing organizations (CMOs)
- No patient biopsy needed
 - Less clinical time and resources
 - Avoids needing biopsy consent from severely ill patients
- Commercial product orientated

• Autologous

- Avoids immune rejection
 - Does not require costly immunosuppression and its associated complications
- May be easier to proceed with, for example, no requirement for cell line development
 - Reduced start-up costs
 - Avoids embryonic sources
 - Simpler regulations
 - Avoids nondonor virus and prion concerns
 - May avoid cell abnormalities given less expansion for individual patient's requirement
- Potential for 'point-of-care' processing
 - Could enable independent clinical technology
- Favored for bioaesthetic applications
- Service model orientated (e.g., embedded in a hospital or clinic)

Table 8: Potential advantages of allogenic and autologous therapeutic cells in regenerative medicine (Table taken from Mason et al.⁷⁹)

Disadvantages

• Allogeneic

- Immune rejection may be a major issue
- Risk of cell abnormalities, particularly with many cycles of in vitro replication
- Teratoma formation risk is a concern
- Provision and consenting of donated cells requires significant time and resources
- Development investment is high

• Autologous

- Variability of source material
- Difficult to generate large numbers of cells from either somatic or adult stem cells
- Inability to deal with emergencies
- Patient throughput will be relatively low
- Difficult to address large numbers of patients at reasonable costs
 - Minimal economies of scale
- Biopsy procedure is not without risk to patients
- Any processing failure involves major treatment delays

Table 9: Potential disadvantages of allogenic and autologous therapeutic cells in regenerative medicine (Table taken from Mason et al.⁷⁹)

Despite successful results of cell based bone tissue engineering approaches *in vitro* and *in vivo*, the transfer of these novel concepts into routine clinical practise has not been realized^{298,299}.

In bone regeneration research, the application of tissue engineering constructs loaded with cells is often transplanted into a bone defect, immediately after the defect was created. Thus, the nutrient and oxygen supply in the defect area is very low and the inflammation reaction is in progress. Therefore, the transplantation of mesenchymal progenitor cells into these bone defect areas often leads to a low survival rate of the cells, resulting in lower bone regeneration.

The regeneration of bone defects starts with an inflammation reaction, followed by the regeneration phase (soft callus phase). The duration of the initial inflammation phase depends on the size of the defect and ends usually

after about 2 - 3 weeks. During the inflammation reaction we can find macrophages and platelets in the defect area, whereas during the soft callus phase this changes to endothelial cells, mesenchymal progenitor cells and chondrocytes³⁰⁰. Hence, the normal appearance of the mesenchymal progenitor cells is after the inflammation reaction. Furthermore an ingrowth of new blood vessels into the defect area has been detected in the soft callus phase, which resulted in a higher nutrient and oxygen supply at this time point. Based on these ideas, our novel concept of delayed injection of mesenchymal progenitor cells into a created large bone defect after 3 – 4 weeks was developed. The better nutrient and oxygen supply at this time point should lead to a higher survival rate of the transplanted cells in the defect area.

So far, our results demonstrate that the concept of delayed cell injection of allogenic mesenchymal progenitor cells leads to bone regeneration with complete bridging of a critically sized segmental defect in a large animal model. The delayed application of allogenic cells is a safe procedure, which didn't show any clinical signs of immune reaction in the animals. We still were not able to find a validated cell labelling method in our large preclinical animal model, to prove the survival rate of the transplanted cells. Therefore, we can't correlate the results regarding bone regeneration in our experimental cell groups to the cells.

In tissue engineering, confluent cultured cells are usually harvested by enzymatic digestion before seeding onto a scaffold. The use of enzymatic digestion in cell culture is a standard procedure and well accepted, but this method separates the cells from the extracellular matrix (ECM) produced

after osteogenic differentiation. This extracellular matrix is rich in growth factors and has a strong osteogenic potential. The Okano group developed the use of cell sheet technologies to allow the cells to recover within their own matrix after transplantation³⁰¹. As we used the cell sheet technology in our study as well, we are not able to distinguish between the osteogenic regeneration potential of our transplanted cells vs. the transplanted cell sheets (ECM). Further analyses have to be made in the future to clearly demonstrate the effect of the transplanted cells.

Nevertheless, the concept of delayed injection of cells for tissue engineering approaches seems to be a promising technique in bone regeneration and might lead to better results in cell-based tissue engineering approaches.

The experimental cell groups have yet to be completely analysed, including histological analyses to get detailed information about the bone regeneration process and to make final conclusions about the delayed cell injection procedure.

Overall Discussion and Conclusions

In regenerative medicine, mesenchymal progenitor cells have emerged as a promising cell source with a great potential for cell-based therapies. It has been shown in several studies that these cells can be isolated from a variety of tissues and can differentiate into several cell lineages like bone, cartilage, tendon, nerve and muscle.

To analyse potential novel bone tissue engineering strategies for a later clinical application in humans, rigorous *in vitro* testing, small animal models and (importantly in the final stages) the use of standardised large animal models are necessary. Thus, large preclinical animal models with comparable body weight, long bone dimensions, similar mineral composition, and equivalent remodelling rates as well as established and standardised evaluation processes are essential to make sufficient predictions about the potential clinical success or failure of new bone tissue engineering strategies.

Over the last decade, the sheep has become a very important and useful preclinical model to address research problems and holds specific advantages compared to other large animal models (e.g. pig, dog) such as long bone dimensions, ease of handling, non aggressive nature, and the ability to keep them in large numbers at relatively low costs.

Our group has established an ovine critical-sized segmental tibial defect model to address different bone tissue engineering approaches by testing combinations of growth factors, scaffolds and different cell types.

Mesenchymal progenitor cells are a common cell source used in cell-based bone tissue engineering approaches. It has been shown in several small animal models, that these cells can be used for allogenic cell transplantation. The supply of autologous MPCs is often limited and the preparations for their isolation, expansion and differentiation is time consuming and expensive. Therefore, the use of an allogenic cell source with comparable regenerative potential as a potential off-the-shelf product would open new avenues of therapeutic intervention for the regeneration of large bone defects.

It has been shown, due to their special immunological characteristics, that MPCs could be used successfully for allogenic cell-based bone tissue engineering approaches^{257,258}.

Our results of the first study (chapter III) demonstrated the positive effect of allogeneic bone marrow-derived mesenchymal progenitor cells (MPC's) on bone regeneration and the clinically safe and easy delivery of these cells into a biodegradable composite scaffold. From our previous studies, knowing that we had developed the most challenging large segmental bone defect model, we didn't expect much bone formation after 3 months. However, we were specifically interested in the safe delivery of the allogenic cells. Because of this we chose this early time point for the first study. We were able to detect a strong trend of more bone formation within the defect in both cell groups (allogenic and autologous MPC) compared to the scaffold only group without significant differences. But it was not clear if the positive effect on the bone formation was due to the cells or the cell delivery vehicle in form of PRP that we were using.

Therefore, we decided for the second study to include a group treated with scaffolds and PRP to determine the differences between the cells and the PRP. As we didn't detect significant differences between the cell groups and the scaffold only group, we decided to include another cell source (osteoblasts) in our study, which is often used in bone tissue engineering. Furthermore, the time point was increased to 6 months in the second study to achieve a higher bone formation within our defect.

Based on previous experiments in our group, it was hypothesized for the second study, that differentiated cells (osteoblasts) have a higher osteogenic potential compared to mesenchymal progenitor cells.

Our results of the second study demonstrated no differences in the osteogenic potential of osteoblast compared to mesenchymal progenitor cells. Furthermore, we couldn't detect significant differences between the scaffold-PRP group compared to the cell groups. This means, we were not able to prove a beneficial effect of the cells for bone regeneration in our experiments so far. The second study showed again a trend of more bone formation and higher biomechanical values for the allogenic MPC group, compared to the osteoblast groups and the scaffold-PRP group. Furthermore, the second study showed again a safe delivery of different allogenic cell sources for bone tissue engineering without any clinical signs of an immune reaction even after 6 months.

Taking the results of study I and study II, the concept of our cell-based tissue engineering approach was discussed and then restructured. Both studies showed a trend of more bone formation in our MPC groups, compared to the

other cell groups (osteoblasts), with respect to the x-ray analyses as well as the microCT analyses.

Furthermore, the easier accessibility and higher proliferation rate of mesenchymal progenitor cells makes them more favourable for novel tissue engineering concepts. Because of this we used only MPCs for our last study. As we could show the safe delivery of allogenic cells for bone tissue engineering in our previous studies, we chose a later time point (12 months) for the third study in order to obtain more information about the bone regeneration from a functional and hence clinical point of view.

One of the unique issues for any scaffold-cell based concept is the interactions between the host and the transplanted cells of the implanted construct. At the implant site, the tissue engineered construct (TEC) would be subjected to inflammatory mediators and signaling molecules such as cytokines, growth factors, and extracellular matrix enzymes and proteins which is different than the native environment of the cells inside the scaffold. Depending on the cell type in the TEC, these mediators could evoke variable responses such as activation, differentiation, proliferation, or migration. Additionally, cells near or on the scaffold surface would be subjected to an environment of low pH, ROS, and degradative enzymes which are specific to the foreign body reaction. Conclusively, TEC's must maintain its properties and functions, and regenerate tissue in the midst of a highly "compromised" environment. In order for the TEC's to perform optimally, specific modulation of the foreign body reaction would be required, however as shown by our results this is an impossible task in a large segmental bone defect.

The delayed cell injection concept in our last study was developed, based on the fact, that after the inflammation reaction slows down, the nutrient and oxygen supply and the vascularity is higher compared to after the defect creation, which should lead to a less compromised environment and hence to a higher cell survival rate and consequently better bone regeneration.

This premise lead to the novel concept of delayed injection of allogeneic mesenchymal progenitor cells into a created large bone defect after 3 – 4 weeks was performed in our last study (study III, chapter V).

The delayed injection of allogeneic mesenchymal progenitor cells lead to complete bridging of critically sized bone defects with massive bone regeneration. The amount of bone formation was comparable to the ABG group when compared using x-ray analyses. X-ray analyses of the last study were performed after 3, 6 and 12 months. Comparing the x-ray results of the first study after 3 months (allogeneic MPC group and scaffold only group) with the x-ray results of the last study (delayed cell injection of allogeneic MPC) we could clearly detect differences with more bone formation in the group with the delayed cell injection after 3 months (Fig. 50).

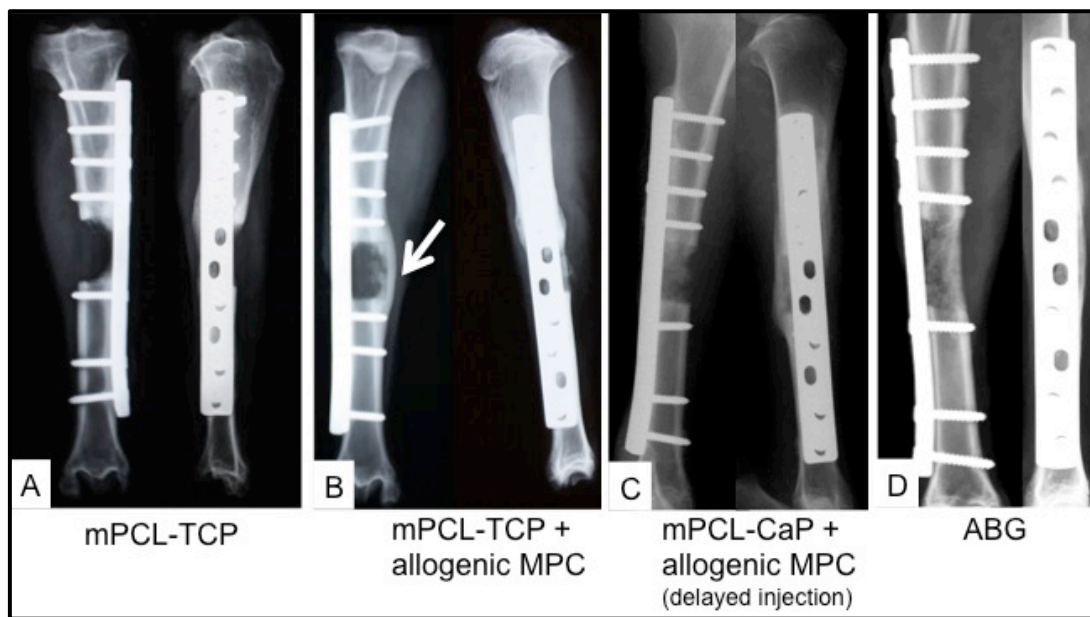


Figure 50: Representative x-ray images, after 3 months, of a defect reconstructed with a mPCL-TCP scaffold (A) and a mPCL-TCP scaffold seeded with allogeneic MPCs (B) (from study I). The x-ray analyses of study I was compared to the x-ray analyses of the cell group with allogeneic MPC from study III (delayed cell injection) (C) and the ABG group of study I (D). The images show clear radiographic signs of bone formation within the defect in the cell group with delayed cell injection (C) as well as the ABG group (D). Notably, there are clear radiographic differences between the cell group of study I (B) and the delayed cell injection group of study III (C) with more bone formation in the delayed injection group (C).

Defining the mechanisms of MSC therapeutic efficacy may require elaborate technology associated with delivery, imaging, and targeting, which has the potential of identifying appropriate delivery mechanisms and the ability to localize hMPCs. Despite the promising results of the delayed cell injection concept so far, all analyses of this experiment (histological evaluation) have to be finished for the final interpretation of the results and the concept. For example, we were still not able to prove the viability of the transplanted cells in our defect. Therefore, to quantify the effect of the transplanted cells, a reliable and validated cell labelling method for large animal models should be used in future experiments.

For further improvement of the delayed cell injection concept and to detect the optimal time point for cell delivery, novel treatment strategies such as the use of contrast-enhanced ultrasound might be used to analyse the perfusion and the blood supply of the bone defect³⁰². The application of biocompatible contrast agents might allow the longitudinal monitoring of the development of the vascularity. Thus, the optimal time point for cell delivery could be determined to achieve a higher cell survival rate and hence the potential for more bone regeneration.

Issues in Understanding scaffold cell mechanism of action <i>in vivo</i>		
Issue	Differences	Approaches
Classification	Define phenotypes and functions.	Negative selection, <u>flow cytometry</u> , <i>in vivo</i> modeling.
Efficacy	Define the success of cells within a study.	Reproducible effects <i>in vitro</i> , <i>in vivo</i> , and clinically.
Potency	How much is enough for cells activity?	Comparison of dosage to endpoint of response.
Mode of administration	Does systemic versus localized administration make a difference?	Direct comparison of localized versus systemic administration in the same model system with the same preparation.
Dosage	How many and how many times do cells need to be administered for effectiveness?	Achieving enhanced efficacy and potency.
Source of MSCs	Fat tissue, amniotic, bone marrow.	Different sources may have different efficacy, potency, and function.
Endpoint of analysis	Inflammation, reparative, regenerative.	Define success of output related to disease of interest.
Cell tracking	Transformation potential, area of impact, local or distance orchestration through the lymphatics	Issues related to tracking for host response and changes in cell phenotype.

Table 10: Issues in Understanding scaffold cell mechanism of action *in vivo* (modified from Dong and Caplan)³⁰³.

In summary, mesenchymal cells both precursors and differentiated exhibit extensive diversity in differentiation, production of trophic mediators, and interaction with the host environment. Clinical studies are ongoing in a variety of indications to determine if the unique functions of hMPCs may have a therapeutic impact on bone regeneration. The issues are that scaffold-cell-based concepts are in their infancy compared to scaffold-BMP based technology, with ongoing studies to define the unique identification of cells in a scaffold, their efficacy *in vivo*, dosage, route of administration, and the duration of their potency. Applications of scaffold-cell-based concepts in large segmental bone defects should be embraced at both the bench and bedside and carried into the clinic to provide new avenues for patient care.

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Appendices

Appendix A – Paper 1

Statement of Contribution of Co-Authors for


The authors listed below have certified* that:

1. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
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Berner A, Reichert JC, Müller M, Zellner J, Pfeifer C, Dienstknecht T, Nerlich M, Sommerville S, Dickinson IC, Schütz MA, Füchtmeier B. Treatment of long bone defects and non-unions: from research to clinical practice. Cell Tissue Res. 2011 May 17.

Contributor	Statement of contribution*
Arne Berner	Wrote the manuscript, experimental design, conducted experiments, and data analysis
Signature 	
Date 18.10.2012	
Johannes Reichert	Aided experimental design, data analysis, Manuscript preparation
Michael Müller	Aided manuscript preparation
Johannes Zellner	Aided manuscript preparation
Christian Pfeifer	Aided manuscript preparation
Thomas Dienstknecht	Aided manuscript preparation
Michael Nerlich	Aided manuscript preparation
Scott Sommerville	Aided manuscript preparation
Ian Dickinson	Aided manuscript preparation
Michael Schütz	Aided manuscript preparation
Bernd Füchtmeier	Aided experimental design, data analysis, Manuscript preparation

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

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Appendix B – Paper 2

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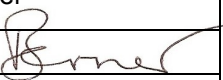
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Berner A, Boerckel JD, Saifzadeh S, Steck R, Ren J, Vaquette C, Zhang JQ, Nerlich M, Guldberg RE, Hutmacher DW, Woodruff MA. Biomimetic tubular nanofiber mesh and platelet rich plasma-mediated delivery of BMP-7 for large bone defect regeneration. Cell Tissue Res. 2012 Jan 26.

Contributor	Statement of contribution*
Arne Berner	Wrote the manuscript, experimental design, conducted experiments, and data analysis
Signature 	
Date 18.10.2012	
Joel Boerckel	Aided experimental design, data analysis, Manuscript preparation
Siamak Saifzadeh	conducted experiments and data analysis
Roland Steck	Aided data analysis and manuscript preparation
Jen Ren	Aided data analysis
Cedryk Vaquette	Aided manuscript preparation
Julia Zhang	Aided data analysis
Michael Nerlich	Aided manuscript preparation
Robert Guldberg	Aided manuscript preparation
Dietmar Hutmacher	Aided experimental design, data analysis, Manuscript preparation
Maria Woodruff	Aided experimental design, data analysis, Manuscript preparation

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Prof. Dietmar W. Hutmacher



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Appendix C – Paper 3

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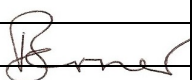
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Berner A, Lam CXF, Arafat MT, Woodruff MA, Saifzadeh S, Steck R, Ren J, Nerlich M, Ekaputra AK, Gibson I, Hutmacher DW. Effects of scaffold architecture on bone healing, submitted to **Biomaterial**

Contributor	Statement of contribution*
Arne Berner	Wrote the manuscript, experimental design, conducted experiments, and data analysis
Signature 	
Date 18.10.2012	
Christopher Lam	Aided experimental design, data analysis, Manuscript preparation and conducted experiments
Muhammad Arafat	Aided manuscript preparation and data analysis
Maria Woodruff	Aided data analysis and manuscript preparation
Siamak Saifzadeh	Conducted experiments
Roland Steck	Aided manuscript preparation
Jen Ren	Aided data analysis
Michael Nerlich	Aided manuscript preparation
Andrew Ekaputra	Aided manuscript preparation
Ian Gibson	Aided manuscript preparation
Dietmar Hutmacher	Aided experimental design, data analysis, Manuscript preparation

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Prof. Dietmar W. Hutmacher



07.02.2013

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Signature

Date

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Appendix D – Paper 4

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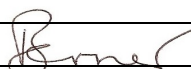
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3. there are no other authors of the publication according to these criteria;
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Berner A, Reichert JC, W, Woodruff MA, Saifzadeh S, Morris AJ, Epari DR, Nerlich M, Schuetz MA, Hutmacher DW. Benchmarking autologous vs. allogenic mesenchymal progenitor cells for the reconstruction of critical-sized segmental tibial bone defects in aged sheep, **submitted to TERM**

Contributor	Statement of contribution*
Arne Berner	Wrote the manuscript, experimental design, conducted experiments, and data analysis
Signature 	
Date 18.10.2012	
Johannes Reichert	Aided experimental design, data analysis, Manuscript preparation and conducted experiments
Maria Woodruff	Aided manuscript preparation and data analysis
Siamak Saifzadeh	Aided data analysis and manuscript preparation
Alyssa Morris	Conducted experiments
Devakar Epari	Aided manuscript preparation
Michael Nerlich	Aided manuscript preparation
Michael Schütz	Aided manuscript preparation
Dietmar Hutmacher	Aided experimental design, data analysis, Manuscript preparation

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Prof. Dietmar W. Hutmacher



07.02.2013

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Benchmarking autologous vs. allogenic mesenchymal progenitor cells for the reconstruction of critical-sized segmental tibial bone defects in aged sheep

*Berner A^{1,2}, Reichert JC^{1,3}, Woodruff MA¹, Saifzadeh S¹, Morris AJ¹, Epari
DR¹, Nerlich M², Schuetz MA¹, Hutmacher DW¹*

*¹Institute of Health & Biomedical Innovation, Queensland University of Technology,
Brisbane, Queensland, Australia*

²Department of Trauma Surgery, University of Regensburg, Regensburg, Germany

*³Department of Trauma, Hand, Plastic & Reconstructive Surgery, Julius-Maximilians-
University, Würzburg, Germany*

Corresponding author:

Dietmar W. Hutmacher
Institute of Health and Biomedical Innovation
Queensland University of Technology
60 Musk Avenue, Kelvin Grove, Queensland 4059, Australia
E-Mail: dietmar.hutmacher@qut.edu.au

Keywords: bone defect, mesenchymal progenitor cells, allogenic, bone
tissue engineering, ovine animal model, bone regeneration

Abstract

Mesenchymal progenitor cells (MPCs) represent an attractive cell population for bone tissue engineering. Their special immunological characteristics suggest that MPCs may be used in an allogenic application. The objective of this study was to compare the regenerative potential of autologous vs. allogenic MPCs in an ovine critical-sized segmental defect model. Ovine MPCs were isolated from bone marrow aspirates, expanded and cultured with osteogenic media for two weeks before implantation. Cells were seeded onto medical grade polycaprolactone-tricalcium phosphate scaffolds (mPCL-TCP) in combination with platelet rich plasma. Autologous and allogenic transplantation was performed by using the cell-seeded scaffolds, unloaded scaffolds and the application of autologous bone grafts served as control groups (n=6). Bone healing was assessed twelve weeks after surgery by radiology, micro computed tomography, biomechanical testing and histology. No local or systemic rejection was observed after transplantation of allogenic cells. Radiology, biomechanical testing and histology revealed no significant difference in bone formation between the autologous and allogenic group. Both cell groups showed more bone formation than the scaffold alone, whereas the biomechanical data showed no significant differences between the cell-groups and the unloaded scaffolds. The results of the study suggest that scaffold based bone tissue engineering using allogenic cells offers the potential for an off the shelf product, which would be desired from a health economic point of view, because such new tissue engineering concepts would drastically lower the therapeutical costs. Therefore, the results of this study serve as an important baseline for the translation of the assessed concepts into clinical application.

Introduction

There are two major engineering approaches in the development of novel treatment concepts using scaffolds; cell-based and cell-free. The most suitable cell source for scaffold-based bone tissue engineering is still focus of much debate in the literature. There is no denying the potential of including a cell population within a tissue engineered construct (TEC) which is able to regenerate the host site; however, the best approach is yet to be determined. Some cell-based strategies aim to improve osteoinduction by the incorporation of cells with a high osteogenic differentiation potential such as bone marrow derived mesenchymal progenitor cells (bMPCs). Gronthos et al.¹ have defined these cells as multipotent progenitor cells, which have the potential to differentiate into a variety of mesenchymal tissues such as bone, cartilage, tendon, ligaments, muscle, fat and dermis.^{2,3,4,5} They can be isolated from a variety of tissues^{6,7, 8} using different separation techniques and can be differentiated into the appropriate phenotype under defined culture conditions and the action of specific growth factors or cytokines.⁹ These cells have shown their therapeutic potential in a number of *in vivo* studies for the regeneration of large bone defects and non-unions.¹⁰⁻¹⁴

The supply of autologous MPCs is often limited and the preoperative preparations for their isolation, expansion and differentiation is time consuming and expensive. Hence, to acquire an adequate amount of cells for transplantation, the time period between cell isolation and cell transplantation is usually at least 4 – 6 weeks. Therefore, the major drawback of using an autologous cell source is two-pronged; limitations in cell numbers when utilising them immediately after extraction or the long time period and associated costs, which are necessary to expand the cells *in vitro* until a suitably high number is attained. However, the special immunological characteristics, which are evident with MPCs, suggest that MPCs could in fact be used successfully for non-autologous applications.^{15, 16} Allogenic cell transplantation is a common therapeutical option and is in routine clinical use in the field of oncology.^{17, 18} Translating the idea of allogenic cell-transplantation from oncology to orthopaedics could offer a new opportunity for the availability of MPCs for regenerative medicine as an “off the shelf product”. Before translating these new treatment concepts into a clinical

application in orthopaedic and trauma surgery, rigorous evaluation of the respective cell populations in an adequate preclinical animal model are essential.^{19, 20}

Several animal models have been developed over the years to verify the practicality of different research approaches in bone regeneration. Among these, adult sheep offer the advantage of having a comparable body weight, a similar mineral composition of bone and similar metabolic and remodelling rates to humans and furthermore, long bone dimensions suitable for the use of human implants and prostheses, which is not possible in smaller species.^{21, 22} Thus, our group has recently established a challenging ovine segmental bone defect model using relatively old animals, which show the secondary osteon remodelling characteristic of human bone. We move towards defining an appropriate cell source for bone tissue engineering to circumvent the aforementioned disadvantages associated with autologous cell transplantation in favour of allogenic MPC sources.²³

We hypothesize that allogenic MPCs have a similar osteogenic potential compared to autologous MPCs and can be used in a scaffold-cell based bone tissue engineering concept. Therefore, the aim of the current study was to assess and compare the regenerative potential of autologous versus allogenic MPCs in combination with a mPCL-TCP scaffold in a critical sized segmental bone defect in a sheep tibia.

Material and Methods

Scaffold fabrication and preparation

Bioresorbable cylindrical scaffolds of medical grade poly-caprolactone (80 wt%) and β -tricalcium phosphate (20wt%), (mPCL–TCP), (outer diameter: 20 mm, height: 30 mm, inner diameter: 8 mm) were produced by fused deposition modelling (FDM) (Osteopore International, Singapore; www.osteopore.com.sg). The structural parameters of the scaffolds were tailored by computer-aided design and included a 70% porosity with 100% pore interconnectivity within a pore size of 350-500 μ m. Filaments of approximately 300 μ m in diameter were deposited following a 0/90° pattern with a separation of approximately 1200 μ m (Fig. 1 F). The scaffolds had an

compressive stiffness of 446.5 N/mm. This architectural layout is particularly suitable for load bearing tissue engineering applications since the fully interconnected network of scaffold fibres can withstand early physiological and mechanical stress in a manner similar to cancellous bone.^{24, 25} Moreover, the architectural pattern allows retention of coagulating blood clots during the early phase of healing, and bone in-growth at later stages. Prior to surgery, all scaffolds were surface treated for six hours with 1M sodium hydroxide (NaOH) and washed five times with phosphate-buffered saline (PBS) to render the scaffold more hydrophilic. Scaffold sterilization was achieved by incubation in 70% ethanol for 5 min followed by complete evaporation and UV irradiation for 30 min.

Biomechanical testing of scaffold and internal fixation

To investigate the mechanical behaviour of the implant-bone-scaffold construct, biomechanical testing was performed *in vitro*. A fixation plate (10 hole Dynamic Compression Plate, Synthes) was affixed to a cylindrical bone analogue cut to a length of 240 mm (fibre-filled epoxy cylinder with a 20 mm outer diameter, 3 mm wall thickness, Pacific Research Laboratories, Washington, USA). A 3 cm defect was created in the bone analogue and a mPCL-TCP scaffold inserted into the defect. The ends of the construct were embedded in Paladur (Heraeus-Kulzer, Hanau, Germany) and the construct mounted in a biaxial materials testing machine (Instron 8874, MA, USA) using a custom-made jig.²⁶ The test area was enclosed and heated to 37 degrees using a patient heater. The specimen was subjected to 5 cycles with the last cycle used for analysis. The custom-made jig was used to simulate three load cases; confined axial compression (500 N), axial torsion (7 Nm) and medial-lateral bending (10 Nm). To determine the interfragmentary movements (IFMs) at the centre of the defect, optical marker rigid bodies (Orthopaedic Research Pins, Northen Digital Inc, Ontario, Canada) were affixed to the proximal and distal fragments of the bone-analogue. The displacements of the rigid bodies were captured via a motion capture system (Optotrak Certus, Northen Digital Inc, Ontario, Canada) and the IFMs calculated using matrix algebra. The test was then repeated with the scaffold removed from the construct.

Cell harvesting

Ovine mesenchymal progenitor cells (MPCs) were obtained from 6-7 years old Merino sheep undergoing experimental surgery. Bone marrow aspirates were obtained from the iliac crest under general anaesthesia (Fig. 1 A). Total bone marrow cells ($5-15 \times 10^6$ cells/ml) were plated at a density of $10-20 \times 10^6$ cells/cm² in complete medium consisting of low glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were subsequently plated at a density of 10^3 cells/cm² (Fig. 1 B). We have demonstrated previously that MPCs express the respective phenotypic profile typical for different mesenchymal cell populations and show a multilineage differentiation potential.²⁷ Two weeks before implantation, the medium was changed to an osteogenic media (DMEM, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 10 µl/ml β-glycerophosphate, 1 µl/ml ascorbic acid and 1 µl/ml dexamethasone) to induce osteogenic differentiation (Fig. 1 C). For 3D cultures, 35×10^6 ovine MPCs suspended in 500 µl of basal medium were mixed with platelet rich plasma (PRP) (described in subsequent section) and then seeded onto each mPCL-TCP scaffold (Fig. 1 G). PRP was activated with thrombin (5 U/ml), and the scaffolds were incubated at 37°C for at least one hour before implantation into the defect.

Cell labelling with Bromodeoxyuridine

Cells from one animal per experimental group were labelled with Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU). Cells for labelling were seeded at a density of 3000/cm² in DMEM/10% FBS and allowed to attach overnight. The day after seeding, BrdU labelling was achieved by incubating ovine MPCs with the BrdU labelling reagent (Invitrogen) at a concentration of 1:100 in DMEM/10% FBS for 6 h. BrdU is a synthetic nucleoside that is an analogue of thymidine. It can be incorporated into newly synthesized DNA of replicating cells substituting for thymidine during DNA replication thus labelling the respective cells. Specific antibodies may then be used to visualize the incorporated chemical.

Preparation of platelet rich plasma

To produce platelet rich plasma (PRP), 80ml of blood was collected from the jugular vein of each sheep and transferred into 3.5 ml monovettes supplemented with sodium citrate (3.8%) at a ratio of 9 volumes blood and 1 volume sodium citrate according to Anitua et al.²⁸ The citrated blood was transferred to falcon tubes and centrifuged in a standard laboratory centrifuge for 20 min at 2,400 rpm (Fig. 1 D/E). Subsequently, the yellow plasma layer from all tubes was transferred to a fresh falcon tube and the platelets were pelleted in a second centrifugation step for 10 min at 3,600 rpm.²⁹ The pellet was resuspended in 1.2 ml of plasma and the platelets counted in a Neugebauer counting chamber. After preparation, the PRP and cell suspensions were loaded onto the sterilized scaffolds (Fig. 1 G).

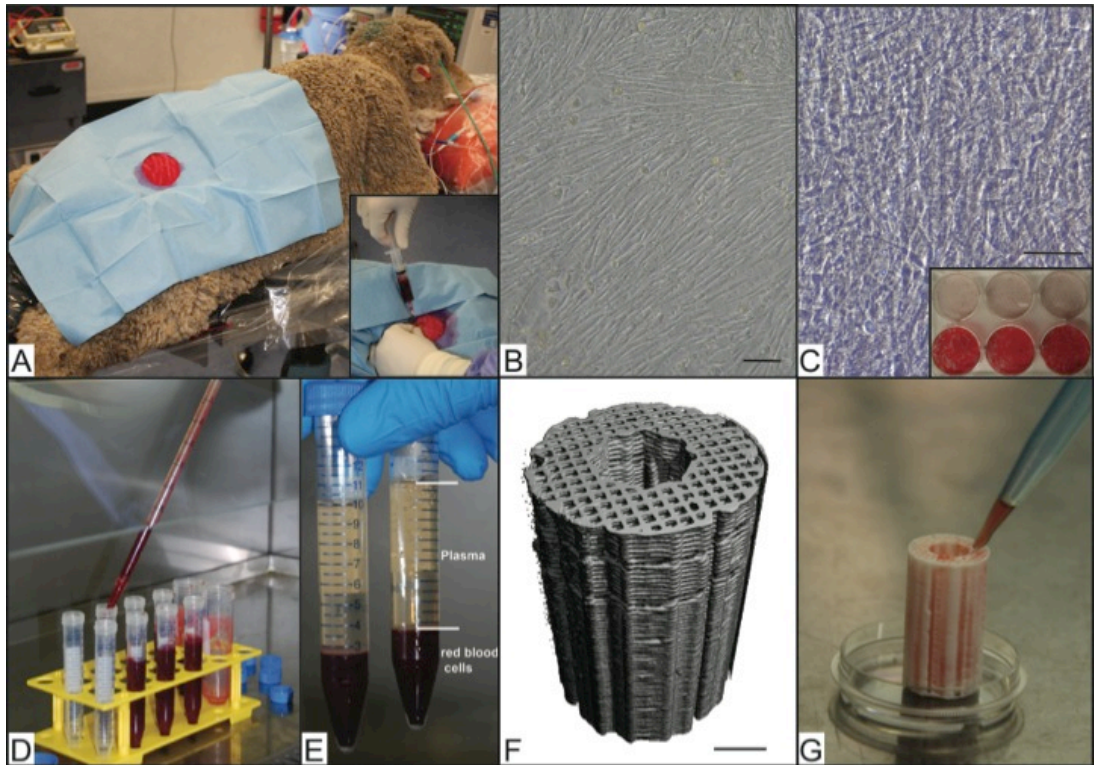


Fig. 1: Bone marrow aspirations (10 ml) from the iliac crest were performed under general anaesthesia (A). MPCs were typically elongated in shape after a culturing period of 10-14 days in expansion media (B) (scale bar=100 μ m). Cell shape changed to a more compact cobblestone-like appearance within days after being exposed to osteogenic media (C) (scale bar=100 μ m). The insert in (C) shows alizarin red staining of MPC cultures after 14 days on 6-well plates. Under osteogenic conditions MPCs secreted a mineralized matrix, whereas the control cultures didn't reveal any staining. To prepare PRP, blood was collected from the jugular vein of the sheep, mixed, and transferred into falcon tubes (D). After centrifugation at 2,400 rpm for 20 min, the plasma was removed and centrifuged a second time (E). The resulting pellet was resuspended in 1.2 ml of plasma, and the cells in combination with PRP were seeded onto the scaffolds (G). A microCT image of the cylindrical mPCL-TCP scaffold produced via fused deposition modelling for segmental bone defect repair is depicted in (F) (scale bar=5mm).

Experimental groups/Surgical procedure

Twenty-four merino sheep (weight 45 ± 2 kg, aged 6-7 years) were operated upon as approved by the University Animal Ethics Committee of the Queensland University of Technology, Brisbane, Australia (Ethics No.: 0900000906). The experimental groups comprised two cell groups (allogenic and autologous cells), a scaffold only group, which served as a negative control, and an autologous bone graft (ABG) group, which served as a positive control. For surgical procedures, all animals were placed in the right lateral position. The right tibia was exposed by a longitudinal incision of approximately 12 cm length on the medial aspect of the limb (Fig. 2 A). A broad dynamic compression plate (DCP, 4.5 mm, 10 holes, Synthes) was adjusted to the morphology of the bone by bending (plate-bending press, Synthes) and applied to the medial tibia. The distal end of all plates were placed 2.5 cm proximal of the medial malleolus (Fig. 2 B). The screw holes were drilled and the plate was temporarily fixed with 2 screws adjacent to the anticipated defect (Fig. 2 C). The middle of the defect site was measured and marked with a raspator. A distance of 1.5 cm each proximally and distally of the defect centre was measured and marked to define the osteotomy lines (Fig. 2 D).³⁰

Next, the soft tissue inserting to the bone in the designated defect area was detached to avoid damage to the proximate nerve and blood vessels during the osteotomy. Parallel osteotomies perpendicular to the bone's longitudinal axis were performed with an oscillating saw (Stryker) under constant irrigation with saline solution to prevent heat-induced osteonecrosis whilst the bone segment of 3 cm length was excised (Fig. 2 E/G). Care was taken to completely remove the periosteum within the defect area and additionally 1 cm proximally and distally of the osteotomy lines (Fig. 2 F, arrow). The plate (DCP) was fixed on the proximal bone using 4 screws. Afterwards, the scaffold was gently placed into the defect and the distal part of the tibia was fixed to the plate using 3 screws (Fig. 2 H/I). One DCP hole was used to place the scaffold under low pressure into the defect. The wound was closed in two layers with a 2-0 Monocryl (Ethicon) and a 3-0 Novafil (Syneture) suture for the skin. The closed wound was sprayed with Opsite (Smith and Nephew), covered with pads and bandaged (Vetrap, 3M).³⁰ After recovery

from anaesthesia, animals were allowed unrestricted weight bearing. After 12 weeks, the animals were euthanized by intravenous injection of 60 mg/kg pentobarbital sodium (Lethabarb, Virbac, Australia).

Group I	Group II	Group III	Group IV
mPCL/TCP, autologous MPC + PRP	mPCL/TCP, allogenic MPC+ PRP	mPCL/TCP	autologous bone graft (ABG)
n=6	n=6	n=6	n=6

Table 1: The four experimental groups of the study. Every group consisted of 6 animals. Group III was treated with a scaffold only (negative control) whereas group IV was treated with ABG (positive control).

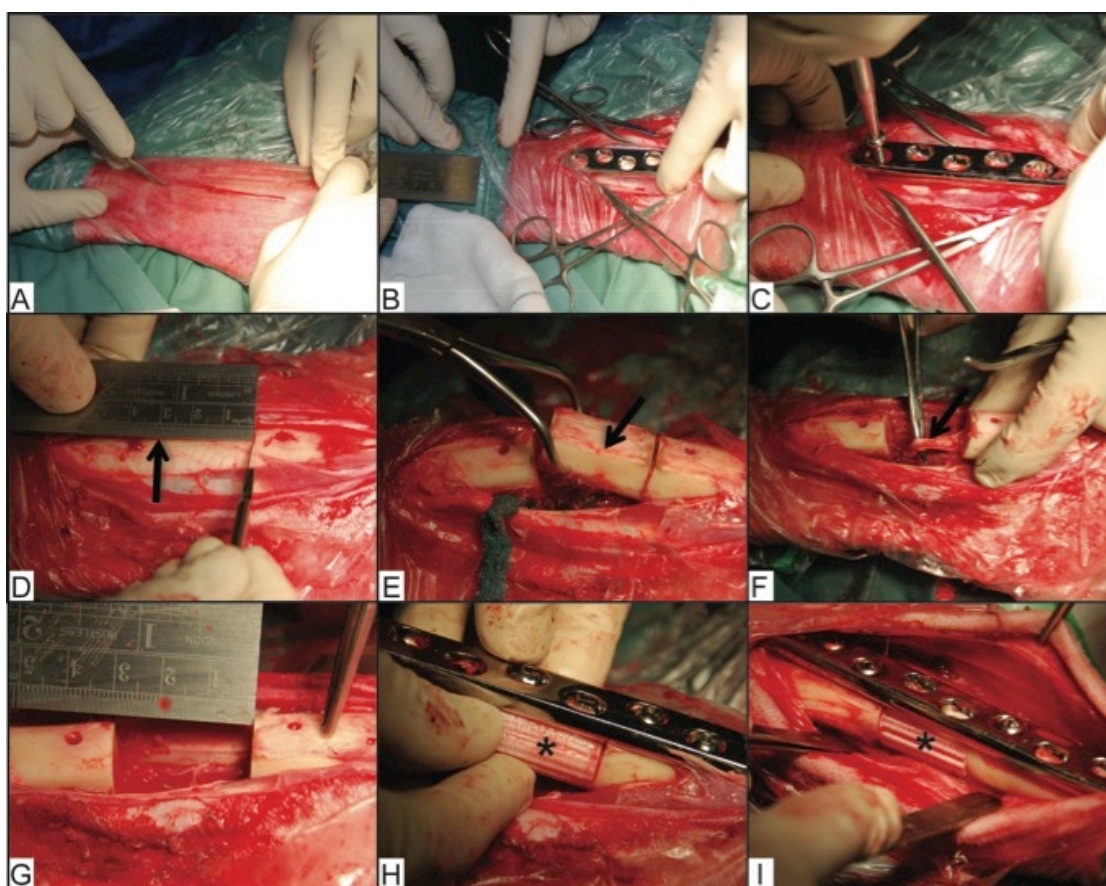


Fig. 2: To create a 3 cm segmental defect, a skin incision was made over the medial part of the tibia (A), the plate was placed 2.5 cm proximal of the medial malleolus (B) and temporarily fixed with two screws (C). The screw holes were drilled and the defect middle and osteotomy lines were marked (black arrow, D). The bone segment was removed after creating a 3cm defect with an oscillating saw (black arrow, E; G). Care was taken to completely remove the periosteum (black arrow, F), which was closely located to the vessels and the nerve in the dorsal part. After implanting the scaffold (*) (H), the bone fragments were realigned and fixed with a plate and screws (I).

Post-operative monitoring

The experimental group with allogenic cell transplantation was monitored for six weeks after the surgical procedure to assess any evidence of an immune reaction to the implantation of the cells. Blood samples were taken from all animals on day 1, 3, 7, 14 and 21 after the operation from the allogenic group and from selected animals from the autologous group as a control to identify any possible immune reactions (white blood cell count). At the same time the sheep were physically examined. A final physical examination was performed six weeks after surgery and animals were observed for the remainder of the study.

Radiographic analysis

Immediately after surgery, and after six and twelve weeks, conventional x-rays were taken to determine the time of bridging of the defect in the different experimental groups. Conventional x-ray analysis (3.2 mAs; 65kV) was performed in two standard planes (anterior-posterior and medial-lateral).

Computed tomography (CT)

ClinicalCT

CT analysis was performed as described previously.³¹ Briefly, a clinical CT scanner (Philips Brilliant CT 64 channels) was used to scan the experimental limbs. A dipotassium phosphate phantom was used to calibrate measurements of mineral density. 3D reconstructions from the CT data were generated with AMIRA® 5.2.2 (Visage Imaging GmbH, Berlin, Germany) (Visage Imaging) with a threshold of 300 and qualitative analysis was performed to assess mineralization within the defect and bridging. For quantitative analysis, the CT datasets of the operated tibia of each animal were edited by using the programs AMIRA® and Rapidform2006 (Inus Technology, Seoul, Korea). The amount of newly formed bone within the 3 cm defect area was calculated using an in-house MATLAB program (MATLAB 7.6.0, MathWorks, Inc., Natick, MA, USA).

Microcomputed tomography (microCT)

MicroCT analyses were performed as described previously.³¹ Briefly,

samples were scanned (μ CT 40, Scanco, Brüttisellen, Switzerland) with a voxel size of 36 μ m. The x-ray tube was operated at 55kV and 145 μ A. Samples were evaluated at a threshold of 210, a filter width of 0.8 and filter support of 1.0 and analysed for bone volume and bone mineral density within the defect using the software supplied by the manufacturer. For the bone volume calculations, the regions of interest were determined as external callus formation (external), bone formation within the scaffold (scaffold) and bone formation in the inner part of the scaffold (endosteal).

Biomechanical testing

Biomechanical testing was performed as described previously.³¹ Briefly, both tibiae of each sheep were explanted and the fixation plate of the experimental leg was carefully removed. Both ends of the tibiae were embedded in 80 ml of Polymethylmethacrylate (PMMA) (Paladur, Heraeus Kulzer, Germany) and each bone was then mounted in an Instron 8874 biaxial testing machine. By leaving as much soft tissue as possible attached, bone samples were prevented from drying out. Next, for the experimental and the intact tibia (control) a torsional test until failure was performed in a biaxial universal testing machine (Instron 8874, Instron, Norwood, USA). The torsion test was conducted with a compressive load of 0.05kN and at an angular velocity of 0.5 deg/s until the fracture point was reached (right tibiae counter clockwise, left tibiae clockwise). In order to avoid the possibility of damage to the specimens in the early stages of bone healing, no preconditioning of the samples was performed. The torsional moment (TM) and torsional stiffness (TS) values were calculated from the slope of the torque-angular displacement curves and normalized against the values of the intact contralateral tibiae.

Histology/Immunohistochemistry

After biomechanical testing and microCT analyses, tibial bone specimens were trimmed to 6 cm length and fixed in 10% neutral buffered formalin for 1 week. For histological analysis, the mid-defect regions were sectioned in the transverse and sagittal plane. The sagittal sectioned samples were used for paraffin embedding. To process the samples for paraffin embedding, bone

samples were decalcified in 15% EDTA for 6-8 weeks at 4 °C. The samples were then serially dehydrated in ethanol in a tissue processor (Excelsior ES, Thermo Scientific, Franklin, MA, USA), and embedded in paraffin. Samples (5 µm) were sectioned using a microtome (Leica RM 2265). The slides were then deparaffinised with xylene and rehydrated before staining with haematoxylin and eosin (Sigma Aldrich) and mounting with Eukitt mountant (Fluka Biochemica, Milwaukee, WI, USA). The remaining samples were used for undecalcified embedding in methylmethacrylate resin (Technovit 9100 NEU, Heraeus Kulzer, Wehrheim, Germany). Longitudinal sections were performed with a thickness of 6 µm and stained with von Kossa/McNeal's Tetrachrome to identify new bone formation and Goldner's Trichrome to identify cellular details. For immunohistochemistry, paraffin sections were deparaffinised with xylene and rehydrated with serial concentrations of ethanol. Subsequently, sections were rinsed in distilled water and placed in 0.2 M Tris-HCl buffer (pH 7.4). Endogenous peroxidase activity was blocked by incubating the sections in 3% H₂O₂ in Tris-HCl for 20 min. This was followed by three washes with Tris buffer (pH 7.4) for 2 min each. Sections were incubated with Proteinase K (DAKO, Botany, Australia) for 20 min and subsequently incubated with 2% bovine serum albumin (BSA) (Sigma, Sydney, Australia) in DAKO antibody diluent (DAKO) in a humidified chamber at room temperature for 60 min to block non-specific binding sites. Afterwards, immunohistochemical staining was performed using primary antibodies specific to the osteogenic markers: type I collagen (Abcam, Cambridge, UK) and osteocalcin (Abcam, Cambridge, UK). The sections were incubated with the specific antibody in humidified chambers at 4°C overnight. Sections were then washed three times for 2 min with Tris buffer (pH 7.4) and incubated with peroxidase labelled dextran polymer conjugated to goat anti-mouse and anti-rabbit immunoglobulins (DAKO EnVision+ Dual Link System Peroxidase, DAKO) at room temperature in humidified chambers for 60 min. Colour was developed using a liquid 3,3'-diaminobenzidine (DAB) based system (DAKO). Kaiser's glycerol gelatin (DAKO) was used for coverslip mounting.

For detection of BrdU labelled cells, a Zymed® streptavidin-biotin based system for BrdU staining (Invitrogen) was used on paraffin sections

according to the manufacturer's protocol.

Statistical analysis

Statistical analyses for the biomechanical results and for the computed tomography scans (clinicalCT and microCT) were carried out using a two-tailed Mann-Whitney-U-test (SPSS 17.0, SPSS Inc.) and p-values are adjusted according to Bonferroni-Holm.

Results

No postoperative infections or other complications were observed in any of the animals. All animals were in good health and survived the experimental period gaining weight in the months following surgery. Notably, the animals of the allogenic group showed no clinical signs of immune response.

Biomechanical testing of scaffold construct (prior to implantation)

Under an axial compression load of 500 N the interfragmentary movement in the defect was 0.27 mm giving an interfragmentary strain ($IFS = IFM/gap\text{-}size$) of less than 1%. There was minimal difference in the IFM (0.21 mm) under compression with the scaffold removed. Subjected to torsion (7 Nm) the construct underwent a relative rotation of the bone fragments of 7.4 deg. Medial-lateral bending induced by an axial load of 100 N at an offset of 10 cm resulted in a shortening of the defect axially by 4.0 mm (IFS 13%) with a bending angle of 1.9 deg.

Monitoring of immune response

Venous blood samples were taken preoperatively and on day 1, 3, 7, 14 and 21 after the operation from all the animals of the allogenic group and some animals of the autologous group (control). The blood tests showed no signs of graft rejection. Preoperatively, the results of the white cell count (WCC) in the allogenic group were slightly higher compared to the autologous group, and did not increase more than in the autologous group after surgery (Fig. 3).

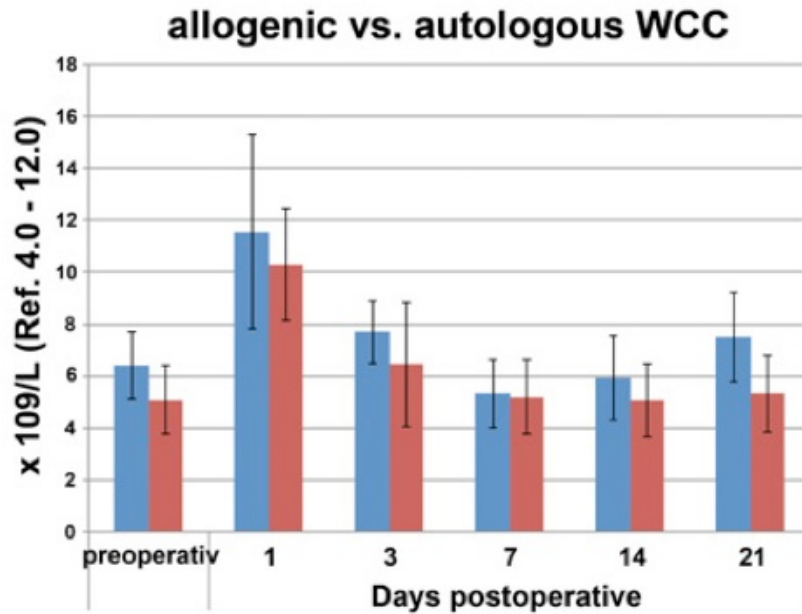


Fig. 3: White cell count (WCC) of venous blood samples of animals from the autologous (blue column) and allogenic (red column) experimental groups. No significant differences were found between the groups.

Radiographic analysis

Immediately after surgery, and after 6 and 12 weeks, conventional x-ray analyses in two standard planes (anterior-posterior and medial-lateral) were performed to assess bone formation. After surgery, the correct position of the scaffold, the plate and the screws were confirmed (Fig. 4 A). After 6 weeks, some initial bone formation was observed starting from the dorsal part of the tibia where the defect was covered by the large muscle of the lower leg (Fig. 4 B). The x-ray analysis after 12 weeks showed no loosening of the implants or movement of the scaffolds. External callus and bone formation within the defect was observed in all animals of the autologous and the allogenic cell group (Fig. 4 C/D). A complete bridging of the defect was only observed in one animal of the allogenic group and two animals of the autologous group.

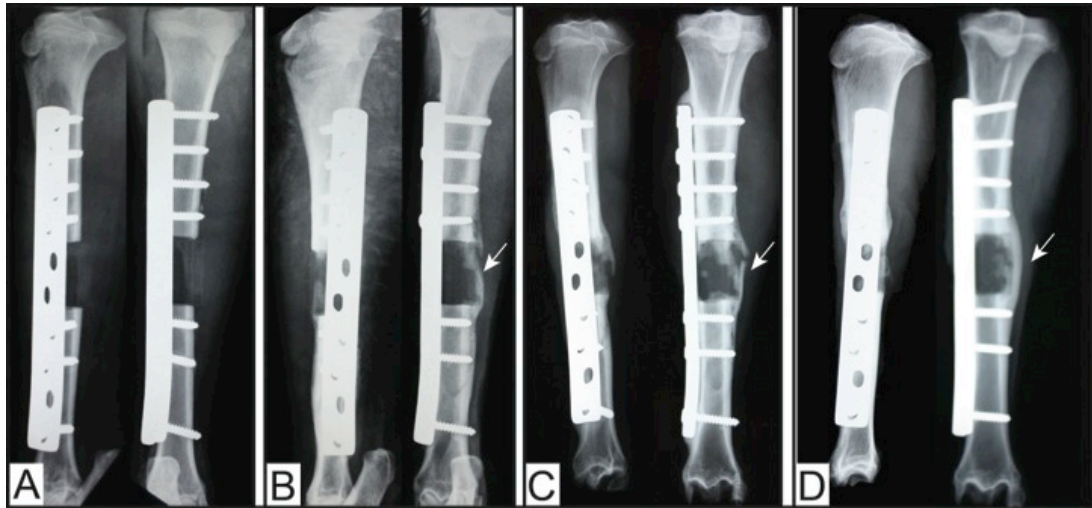


Fig. 4: Representative x-ray images directly after surgery (A), after 6 weeks (B) and after 12 weeks (C) of defects reconstructed with mPCL-TCP scaffolds seeded with autologous MPC's (A-C) and with allogenic MPC's (D). The images show clear radiographic signs of a beginning defect bridging in both cell groups (white arrow) on the site of the vascular bundle (enhanced vascularity).

Computed tomography (CT)

ClinicalCT

Qualitative CT analysis after 12 weeks showed bone formation within the defect in both experimental cell groups (Fig. 5 A, B). Minor bone formation was seen in the mPCL-TPC scaffold group (negative control group), whereas a full defect bridging had occurred in all defects reconstructed with ABG (positive control group). No radiographic signs of inflammation (e.g. diffusely delimited soft tissue infiltrations, osteolysis, osteomyelitis, abscesses) were found. Scaffolds showed good osseointegration without any signs of resorption. Median values of total bone volume (BV) in the defect were higher in the allogenic cell group compared to the autologous cell group. Furthermore, both cell groups showed a higher total bone volume compared to the scaffold only group (Fig. 5 G). However, the differences were not statistically significant.

MicroCT

MicroCT analysis confirmed the trends observed with the clinical CT scans regarding union rates and the amount of new bone formation (Fig. 5 C, D). In both cell groups, the mean values of newly formed bone were higher compared to the scaffold only group (Fig. 5 E). Highest median values of newly formed bone were found for the ABG group, which were significantly

higher compared to the scaffold only group, but were not significantly different to both cell groups (Fig. 5 E). However, even in the ABG group, newly formed bone still only reached levels equating to 50% of that determined for the same anatomic level of the contralateral hind limbs (control) (Fig. 5 E). The amount of new bone was distributed equally throughout the external, scaffold and endosteal area of the defect (Fig. 5 F). A tendency towards a higher volume of bone formation in the allogenic cell group compared to the autologous cell group and the scaffold only group was observed (Fig. 5 F). The tissue mineral density of the regenerated bone showed similar results in all experimental groups of approximately 60 – 80% of the contralateral tibia (Fig. 5 H).

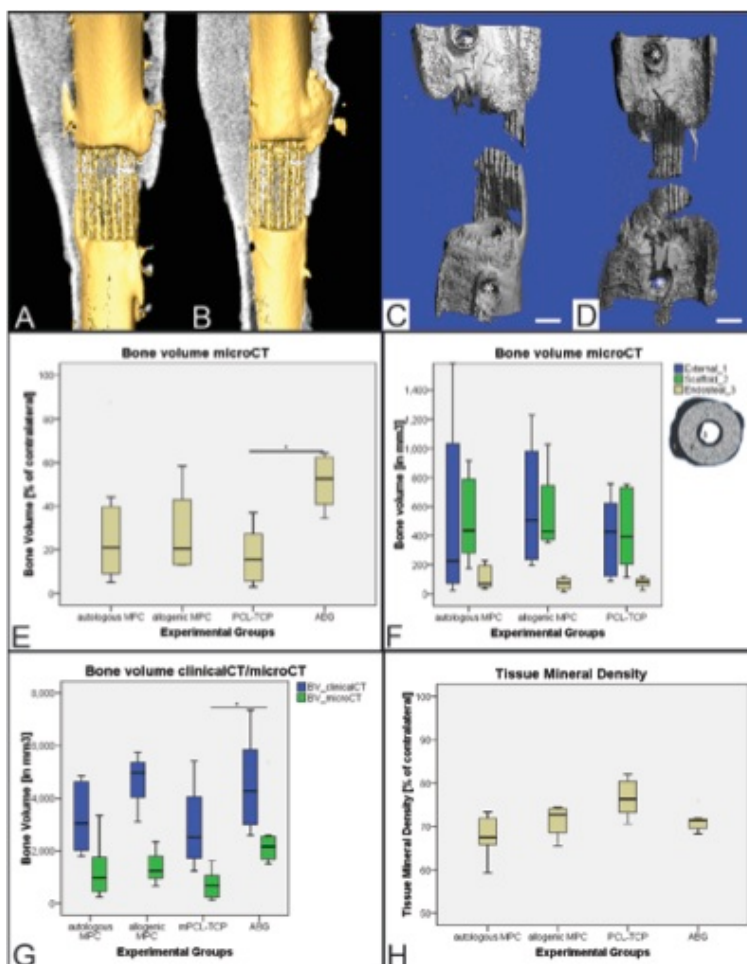


Fig. 5: Representative 3D clinicalCT data reconstructions (AMIRA 5.2.2) of critical segmental bone defects showed a similar bone formation within the defect in the autologous cell group (A) and the allogenic cell group (B). The 3D reconstructions of the clinicalCT scans were performed with a threshold of 300 and combined with a 2D reconstruction using a lower threshold to show the soft tissue and the scaffold. Box plots demonstrating the median \pm 1st and 3rd quartile. Error bars represent minimum and maximum values. MicroCT 3D reconstructions showed just a small amount of bone formation within the defect in the autologous cell group (C) (bar=5mm) as well as the allogenic cell group (D) (bar=5mm). The drill holes in the host bone, through which the screws inserted to attach the DCP plate may

be observed in (C) and (D), (white asterisk). The microCT results depict the median amounts of newly formed bone within the 3 cm defects as a percentage of the contralateral control side (E). The amount of bone volume was distributed into external part (1), scaffold area (2) and the endosteal area (3). Both cell groups showed a slightly higher amount of bone formation within the scaffold area compared to the scaffold only group (F). The total bone volume (BV) after 12 weeks, as measured by clinicalCT and microCT, showed slight differences in the absolute values due to the different resolution of both scanning methods (G). The tissue mineral density of the regenerated bone in all experimental groups was approximately 60 – 80% of the contralateral tibia (H).

Biomechanical testing

Biomechanical testing was performed on all operated groups (right tibiae) as well as the non-operated left tibiae, which served as a control (Fig. 6 A). Biomechanical testing revealed an equal torsional stiffness (TS) for the autologous and the allogenic cell groups (Fig. 6 B) and slightly higher values for the torsional moment (TM) for the autologous group (Fig. 6 C). Both cell groups showed a higher torsional moment, but a comparable torsional stiffness compared with the scaffold only group. The samples of the ABG group showed the highest values for torsional moment and torsional stiffness, however they only reached approximately 15 to 20% of that obtained from the contralateral non-operated side (control). No significant differences were found between all the groups by biomechanical testing.

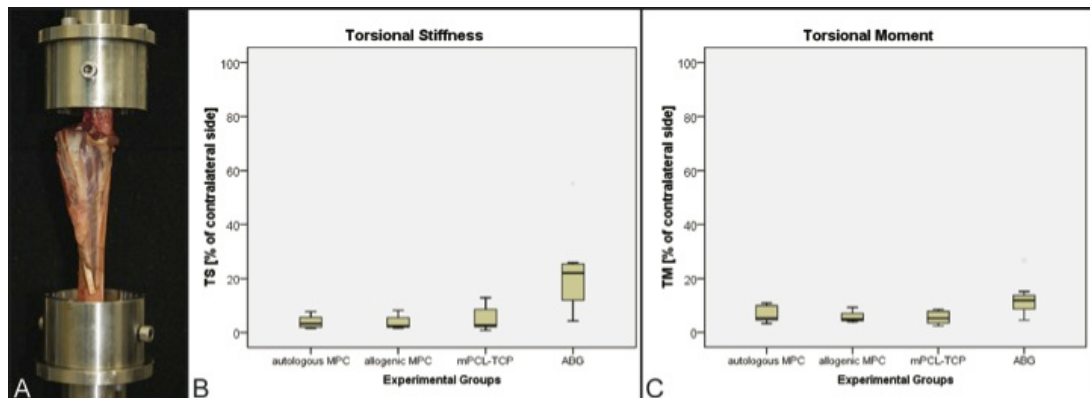


Fig. 6: For biomechanical testing, both ends of the tibia were embedded in Polymethylmethacrylate with the tibial axis vertically aligned (A). Box plots demonstrating median values \pm 1st and 3rd quartile of torsional stiffness (B) and torsional moment (C) in relation to the contralateral tibia. Error bars represent maximum and minimum values. Both cell groups showed a higher torsional moment, but a comparable torsional stiffness compared with the scaffold only group. The samples of the ABG group showed the highest values for torsional moment and torsional stiffness.

3.4. Histology/Immunohistochemistry

The macroscopical overview of the implanted scaffolds before processing for histology showed a good integration of the scaffold to the host bone in all animals (Fig. 7 A). The mPCL-TCP scaffold was still in place and had not resorbed. Histological examinations of decalcified samples were performed after 12 weeks. Representative H&E staining of both cell groups demonstrated a good integration of the scaffold to the host bone on the proximal as well as the distal side (Fig. 7 B, C, F, G). Notably, new bone formation was seen in many pores within the mPCL-TCP scaffolds in both cell groups (the scaffold itself is revealed in the histology slices as empty circular “holes” and “bars” where the scaffold struts used to reside, due to the dissolution of the mPCL implant by xylene during processing) (Fig. 7 B-G).

Undecalcified sections (MMA resin) were stained with von Kossa/McNeal's to identify new mineral deposition. Both cell groups showed mineral deposition (black stain) within the defect (Fig. 8 B, G). These results confirm our microCT and clinicalCT evaluation and demonstrate a limited amount of bone tissue growing into the whole scaffold area. Furthermore, the large overview pictures of the Goldner's Trichrome staining demonstrate the presence of collagen fibres in the scaffold and surroundings (Fig. 8 A, F). New bone formation is traditionally accompanied by the expression of bone specific proteins within the extracellular matrix surrounding the osteoblasts, this may be detected using immunohistochemistry. As an early non-specific marker of osteoblastic differentiation during mineralisation, type I collagen showed a similar intensity in both cell groups (Fig. 8 D, I). Immunohistochemical staining for osteocalcin also demonstrated similar expression levels with a high expression of this late osteogenic marker localised around the scaffolds struts (labelled “s”) (Fig. 8 E, K). The staining to detect the BrdU labelled cells did not reveal any evidence of survival of the donor cells (data not shown).

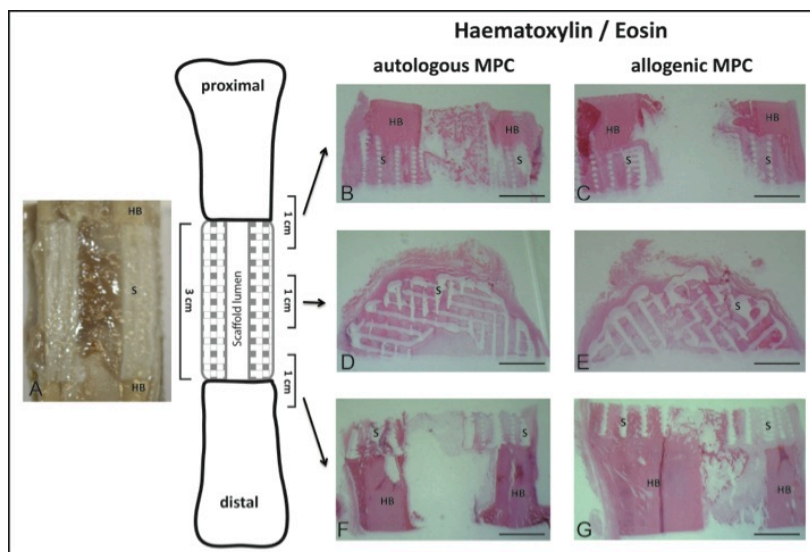


Fig. 7: Macroscopic overview of the scaffold/cell constructs within the defect after explantation (A). Representative H&E staining of both cell groups of the proximal (B, C) (longitudinal sections), middle (D, E) (transverse sections) and distal (F, G) (longitudinal sections) parts of the defect showed a good integration of the scaffold (S) as well as a good bonding of the regenerated bone to the host bone (HB), (B-G; bar=0.5cm). The solvents used during the preparation of the histological sections resulted in the mPCL–TCP scaffold material being dissolved during embedding. Hence mPCL–TCP struts (S) are represented in histological sections as empty “holes” of similar geometry.

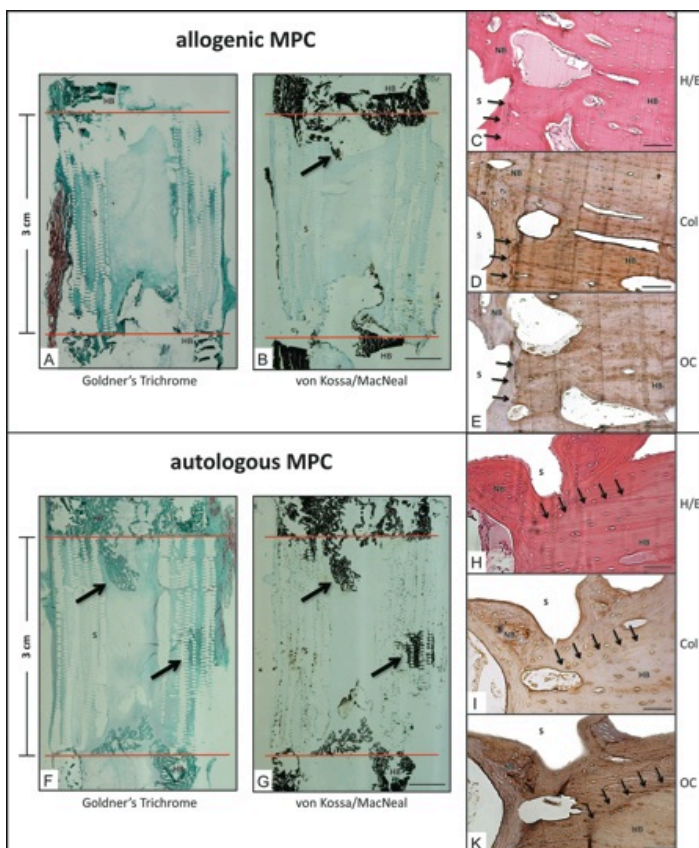


Fig. 8: Overview pictures of the undecalcified resin-embedded samples, sectioned and stained with von Kossa/McNeal's (B, G; bar=0,5cm) and Goldner's Trichrome (A, F) demonstrated the mineralized tissue within collagen fibres (black arrows). Decalcified samples were stained with H&E (C, H), collagen type I antibody (D, I) and osteocalcin antibody (E, K); (bar in C-E, H-K=100µm). Representative stainings of both cell groups

showed a good integration of the scaffold (scaffold strut labelled s) as well as a good bonding of the regenerated bone (NB) to the host bone (HB)(black arrows in E, H, I and K). The solvents used during the preparation of the histological sections resulted in the mPCL–TCP scaffold material being dissolved during embedding. Hence mPCL–TCP struts (S) are represented in histological sections as voids of similar geometry.

Discussion

The use of allogenic cells for clinical therapy is already established and in routine use in other areas of medicine such as oncology and a large number of clinical trials are currently performed in cardiology.^{32, 33} Our results demonstrated the positive effect of allogenic bone marrow-derived mesenchymal progenitor cells (MPC's) on scaffold-based bone regeneration. In addition to their osteogenic potential, it has been shown that MPCs are immunologically privileged making them highly appropriate for the use in allogenic cell transplantation concepts, and indeed we observed no immune response in a preclinical animal model. The allogenic application of MPCs showed similar results for bone regeneration compared to the autologous cell group, with all the advantage of an allogenic cell source (easy to access and high abundance compared to autologous cells). Our results are in line with observations from Guo et al. who described that allogenic mesenchymal progenitor cells raised a minimal immunological reaction only in the early stages after implantation in a study in mini-pigs.³⁴ After the transplantation, the sheep were monitored by clinical examination and taking blood samples. We did not observe any general foreign body reaction or cell rejection or any different cellular reactions compared to the autologous group in the histological assessment throughout the defect, which demonstrated, once more, the low immunological activity of allogenic bone marrow derived cells. While the limited availability and the donor site morbidity of autologous bone grafts is often discussed and stated as a major disadvantage of this technique, the use of an allogenic cell source with comparable regeneration potential as a potential off-the-shelf-product would open new routine therapeutic potentials for regeneration of large bone defects.^{35, 36} Niemeyer et al. used human MPCs to assess bone regeneration in a critical sized defect of the sheep tibia and compared the regenerative potential with autologous ovine MPCs.¹⁵ The autologous MPCs demonstrated better bone

formation compared to the human MPCs and unloaded matrices in the histological and radiological evaluation. However, the bone regeneration was evaluated based on two dimensional imaging procedures and the study lacked any biomechanical testing, which would provide a greater depth of understanding of the characteristics and functionality of the regenerated bone. Field et al. evaluated the efficacy of allogenic mesenchymal progenitor cells for the repair of an ovine tibial segmental defect.¹⁴ They reported a higher osteogenic regeneration potential of the allogenic group compared to the scaffold only group with better biomechanical properties and more bone formation. But, studies by Field et al. and Niemeyer et al. used different fixation methods (double plate vs. intramedullary nail) and different experimental protocols, which renders direct comparison of these findings difficult. Using a medullary nail for example will result in blocking the medullary cavity of the tibia, which might reduce the regeneration potential in this area. Furthermore, different fixation methods result in different biomechanical impacts, such as more flexibility and a central loading (nail) compared to a much stiffer double plate fixation. Our biomechanical analysis of the DCP-plate fixation showed, under static loading conditions of up to 500 N, a minimal displacement of the scaffold of less than 1 %. Conclusively, the DCP results in a stable fixation. From an ethical as well as an economical point of view, it would be desirable to be able to directly compare the potential of different tissue engineering strategies to lower the costs and the number of animals and to realise the translation of novel concepts from research to clinical practice. Therefore, the bone tissue engineering community should advocate the combination of radiological, biomechanical, histological and immunohistochemical evaluations as necessary methodologies for an efficient and robust analysis of bone engineering strategies.³⁷

As bone tissue engineering has become ever more prevalent over recent years, evaluating the different strategies with respect to potential clinical application in humans requires *in vitro* testing and small animal models and importantly, in the final stages, the use of standardised large animal models which are imperative for rigorous preclinical evaluation.³⁸ Thus, large preclinical animal models with comparable body weight, long bone

dimensions, similar mineral composition, equivalent remodelling rates as well as established and standardised evaluation processes are essential to make sufficient predictions about the potential clinical success or failure of new bone tissue engineering strategies.^{19, 20} Over the last decade, the sheep has become a very important and useful model to address research problems and holds specific advantages compared to other large animal models (e.g. pig, dog) such as long bone dimensions, ease of handling, non aggression nature, and the ability to keep them in large numbers at relatively low costs. However, it is important to note that secondary bone remodelling is only seen in older sheep making them comparable to human bones.³⁹ Our group has established an ovine critical-sized segmental tibial defect model to address different bone tissue engineering approaches by testing combinations of growth factors, scaffolds and different cell types.^{20, 23} Compared to studies by other groups, this current model is a particularly challenging model using older sheep which are on the one hand more relevant to human bone but on the other hand have a lower general health status compared to younger animals. Some other groups only resect the periosteum on the proximal and the distal bone, however, we have shown in a preliminary study, that the periosteum on the dorsal side between the tibia and the muscles which is closed to the vascular bundle (see back arrow Fig. 2F) contains a significant regeneration potential based on the MPCs from the cambium layer.²³ To our knowledge, we are the first group who developed a large segmental tibial defect model, which also includes the removal of this part of the periosteum, which represents 30-40% of the whole volume of the periosteum within the defect. As a result of the complete removal of the periosteum and the age-related compromised regenerative capacity, it is not surprising that we revealed lower biomechanical stability (TM 10-15% and TS 20-25% of contralateral tibia) after three month even in the ABG group compared to other research groups who even present significantly higher results in their control groups.^{40, 41} This demonstrates once again the importance of standardised large animal models with validated experimental protocols to compare outcomes of each different bone engineering approach. This is a *condition sine qua non* to move concepts from bench to bedside.

The present study showed effectively that allogenic MPCs can be safely used in combination with a mPCL-TCP scaffold for bone regeneration in a critical sized bone defect in a sheep model. None of the animals of the study showed a rejection of the TECs or a foreign body reaction in form of a fibrous encapsulation. The allogenic and the autologous cell groups showed comparable results with respect to biomechanical properties and new bone formation. The biomechanical evaluation showed a small difference between the cell groups and the scaffold-only group, whereas the bone volume analysis using clinicalCT and microCT showed larger differences between these groups. This is likely due to the short time frame (three months) we chose for our experiment to terminate. We detected more bone volume in both cell groups compared to the empty scaffold groups, which demonstrated that autologous as well as allogenic cell transplantation leads to enhanced bone formation in a critical sized segmental defect model. But within the three month time period, the bone regeneration was still in its early progress phase and most of the defects were not completely bridged which resulted in low biomechanical test results. Most other studies using a critical sized segmental tibial defect in a large animal had longer time frames for their experiments, which logically resulted in more bone formation and higher biomechanical stability.¹⁴ As we were specifically interested in considering the immune response and the safety of delivering allogenic MPCs at this stage of our research we intentionally chose an early time point.

A common practice to improve the biological competence of osteoconductive scaffolds is via incorporation of osteogenic stimuli such as bone morphogenetic proteins (BMP) and/or vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF). Platelet rich plasma (PRP) consists of several growth factors including significant amounts of VEGF and PDGF and has been in clinical use for many years in the dental surgery field. However the benefits of PRP are still ambiguous regarding large bone defect regeneration in orthopaedics, whereas the positive effect of BMPs for bone regeneration has been shown extensively in several small and large animal models as well as in the clinic.⁴²⁻⁴⁶ Because of the similarity to clinically used fibrin glue, PRP converts from a liquid state into a fibrin hydrogel in the solid state after being activated by thrombin and therefore it is

used in several studies as a highly biocompatible cell-loading vehicle.⁴⁷ We could demonstrate that the scaffold-PRP construct used in our study for large bone defects didn't have significant effects regarding bone regeneration when compared to the scaffold only group (unpublished data). But it can be used as a very effective cell delivery vehicle in a scaffold design with a channel-like architecture with large pores and large pore interconnections which allow full vascularization in a 3 cm segmental bone defect.

The evaluation of the bone volume using both clinical- and micro-CT scanning showed slight differences in the raw data (absolute values) due to the different resolutions as well as the different phantoms used for calibration; however the trend was the same. The experimental groups all showed a comparable tendency with respect to the contralateral tibia of the animals (Fig. 5 F). Keeping in mind the concept of translational research which might result in a later clinical application, the assessment of the correlation between clinicalCT scanning and microCT scanning becomes important, especially for future interpretation of clinicalCT scanning results from large bone defects in humans, as these may be directly related to the CT-results of the large animal studies. Beside the CT-scanning, which is essential for the 3D-reconstruction overview and analysis of the bone volumes, the histological evaluation plays an important role for a detailed evaluation on a cell-based level. Therefore, both evaluation methods (CT-scanning and histology) are absolutely required to perform a reliable study to analyse different tissue engineering approaches for bone regeneration.

The use of cell-based tissue engineering strategies is often compromised due to a low survival rate of the implanted cells. To prove the effect of the transplanted cells, different labelling methods are available to demonstrate the survival of the transplanted cells at the end of the experiment.⁴⁸ Among these, BrdU-labelling is a straightforward method of cell labelling showing good results in small animal models. However, despite the potential shown using small animal models, we were not able to detect any BrdU-labelled cells in the large bone defect model.²⁷ This may be due to labelling the cells in passage 1 and using the cells of passage 3-4 for the *in vivo* experiments, because of the high amount of cells required for the experiment, hence the proliferation of the cells may have led to a loss of signal. Li et al. reported a

decreasing of the labelling signal of BrdU over time, describing that the percentage of BrdU-positive cells decreased from 94% in passage 0 down to 18% in passage 2.⁴⁸ Therefore, for the use in large segmental bone defects such as our sheep model, another labelling method would be more appropriate.^{49, 50}

Furthermore, a detailed and critical understanding of using allogenic cells for bone tissue engineering would be desirable, but can't be easily achieved from a histological perspective when using ovine cells, because of a lack of specific ovine antibodies. These precise questions could be answered by using human cells in a xenogenic transplantation setting, but as Niemeyer et al. demonstrated, human MPCs led to reduced bone formation in a ovine defect model compared to the use of allogenic cells.¹⁵

The present study showed promising data using an allogenic compared to autologous cell source for regeneration of critical sized segmental bone defects in a large animal model. The use of allogenic or autologous cells combined with a mPCL-TCP scaffold showed no differences in their bone regeneration potential as demonstrated by radiological, histological and biomechanical results. We furthermore detected no adverse immunological response. In the future, the successful translation of allogenic cell transplantation into clinical practice could provide beneficial treatment alternatives for challenging bone defects.

Acknowledgements

The authors thank Edward Jiongyu Ren and Linnea Bergenholm for their help with the preparation of the histological specimens, Dr. Ferry Melchels and Dr. Roland Steck for μ CT assistance and Dr. Amaia Cipitria for advice on μ CT and histology technologies. This work was supported by funding through the Australian Research Council (ARC), the AO Foundation and the German Research Foundation (DFG)(BE 4492/1-2).

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