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Development of Biomaterials and Cell Therapies for Bone Regeneration

José Miguel Campos

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

JOSÉ MIGUEL FRANCO ESTEVES DE CAMPOS

DEVELOPMENT OF BIOMATERIALS AND CELL THERAPIES FOR BONE REGENERATION

Thesis for the degree of Doctor in Veterinary Sciences submitted to the Abel Salazar Institute of Biomedical Sciences of the University of Porto.

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"PhD - Doctor of Philosophy Thesis
to the Degree of Doctor in Veterinary Sciences."

“Perfection is lots of little things done well.”

Fernand Point

Resumo

O recurso a enxertos ósseos é preconizado em inúmeras cirurgias. O autoenxerto é aceite como a técnica de eleição na fusão óssea, de defeitos críticos e não-críticos, dadas as suas propriedades de osteoindução, osteocondução e osteogénese. Independentemente destas características exclusivas, verifica-se a crescente substituição pelo uso de osso sintético, devido à alta morbilidade associada à colheita de osso e às limitações no volume desses autoenxertos colhidos. Também as desvantagens associadas aos aloenxertos e xenoenxertos, como o risco de transmissão de doenças infeciosas e a indução de reações imunológicas adversas conduzem à tendência do enxerto de osso sintético.

O Bonelike® é um exemplo de enxerto ósseo sintético com propriedades osteoindutoras e osteocondutoras, que supera algumas das principais desvantagens da tradicional hidroxiapatite por ser composto por uma matriz modificada de hidroxiapatite com as fases secundárias do fosfato tricálcico α e β, possuindo ainda iões que são parte mineral do osso e conhecidos por aumentar a taxa de regeneração óssea, tais como o sódio, o flúor e o magnésio. Este substituto ósseo apresenta uma maior solubilidade do que os biomateriais apenas constituídos por hidroxiapatite. Este facto permite criar melhores *perfomances* osteoindutoras e osteocondutoras, que já foram confirmadas em modelos experimentais (ovino) e aplicados pela primeira vez em casos clínicos de animais de companhia durante este trabalho de doutoramento, verificando-se uma boa resposta radiográfica com aumento da radiopacidade nos locais de defeitos preenchidos com Bonelike® com significativa melhoria clínica dos animais.

Atualmente a aplicação clínica de um biomaterial prevê a sua importante capacidade de combinação às terapias celulares como veículo celular seguro e eficiente. O uso de terapias com base nas células estaminas mesenquimatosas em Medicina Veterinária e Humana é baseado na qualidade e eficiência destas células em modular localmente a resposta inflamatória, produzir fatores de crescimento, substituir células danificadas e promover a vascularização, com vista a otimizar a regeneração dos tecidos. O desenvolvimento pré-clínico de produtos baseados em células estaminas mesenquimatosas inclui o uso de populações de células bem caracterizadas, cuja eficácia e segurança são testadas *in vitro* e em modelos animais apropriados, antes de ensaios e aplicações clínicas.

Neste sentido, no presente estudo, isolaram-se células estaminas mesenquimatosas da polpa dentária de dentes humanos, sendo posteriormente expandidas e caracterizadas *in vitro*, para aplicação *in vivo* associadas ao substituto ósseo sintético Bonelike®. Técnicas como a citometria de fluxo, análise imunocitoquímica, análise cariotípica, perfil metabólico e análise de secretoma foram utilizadas para a caracterização das células estaminais mesenquimatosas da polpa dentária humana isoladas e expandidas *in vitro* previamente á sua aplicação no modelo animal ovino. Foi ainda avaliada a sua capacidade de diferenciação em três linhagens, nomeadamente, osteogênica, condrogénica e adipogénica.

O potencial osteogénico das células estaminas mesenquimatosas da polpa dentária de dentes humanos derivadas da nova metodologia descrita no presente trabalho experimental, associado ao potencial osteoindutor e osteocondutor do Bonelike®, foi testado *in vivo* no modelo ovino pela primeira vez e demostrou uma melhoria nos resultados histomorfométricos e de osteointegração dos defeitos ósseos, promovendo de forma significativa a regeneração óssea.

Este trabalho de Doutoramento destaca ainda a importância de incluir uma equipa multidisciplinar no desenvolvimento de novos biomateriais e terapias baseadas em células, permitindo uma estreita participação de conhecimento entre o desenvolvimento de novos biomateriais e das terapias celulares, o aperfeiçoamento das técnicas cirúrgicas, onde a Medicina Veterinária assume um papel fundamental nos ensaios pré-clínicos para terapias humanas, mas também, nos ensaios *in vivo* para aplicação dessas mesmas terapias em espécies domésticas, nomeadamente animais de companhia e equinos.

Palavras-chave: Biomaterial, terapias celulares, regeneração óssea, enxerto osséo sintético, Bonelike®, células estaminais, células estaminais mesenquimatosas da polpa dentária humanas, modelo experimental ovino, animais de companhia, ensaios pré-clínicos.

Abstract

The use of bone grafts is recommended in innumerable surgeries. Autograft is accepted as the technique of choice in bone fusion, of critical and non-critical defects, given its osteoinduction, osteoconduction and osteogenesis properties. Regardless of these unique characteristics, there is a growing substitution for the use of synthetic bone, due to the high morbidity associated with bone harvesting and the limitations in the volume of these collected autografts. Also the disadvantages associated with allografts and xenografts, such as the risk of transmission of infectious diseases and the induction of adverse immunological reactions, lead to the tendency of the synthetic bone graft.

Bonelike® is an example of a synthetic bone graft with osteoinductive and osteoconductive properties, which overcomes some of the main disadvantages of traditional hydroxyapatite because it is composed of a modified matrix of hydroxyapatite with secondary phases of α and β tricalcium phosphate, but also its composition in characteristic ions of the mineral part of the bone which are known to increase the rate of bone regeneration, such as sodium, fluoride and magnesium. This bone substitute exhibits greater solubility than the single hydroxyapatite type biomaterials. This fact allows the creation of better osteoinductive and osteoconductive perfomances, which have already been confirmed in experimental models (ovine) and applied for the first time in clinical cases of companion animals during this doctoral work, with a good radiographic response revealing increased radiopacity associated to the presence of Bonelike®, with significant clinical improvement of the treated animals.

Nowadays, the improvement of a biomaterial predicts its association to cellular therapies as a safe and efficient cellular vehicle. The use of mesenchymal stem cell-based therapies in Veterinary and Human Medicine is based on the quality and efficiency of these cells in locally modulating the inflammatory response, producing growth factors, replacing damaged cells and promoting local vascularization, in order to optimize tissue regeneration. Preclinical development of mesenchymal stem cell-based products includes the use of well-characterized cell populations whose efficacy and safety are tested *in vitro* and in appropriate animal models prior to assays and clinical applications.

In this sense, in the present study, mesenchymal stem cells were isolated from the dental pulp of human teeth, expanded and later fully *in vitro* characterized, for *in*

vivo application. Techniques such as flow cytometry, immunocytochemical analysis, karyotype analysis, metabolic and secretory profile and tri-lineage differentiation into osteogenic, chondrogenic and adipogenic lineage were use d to characterize de cell-therapies developed by the viable and reproducible method that was standardized and described in this thesis.

The osteogenic potential of the mesenchymal stem cells from human dental pulp were associated to Bonelike® to improve its osteoinductive and osteoconductive potential and this association was tested *in vivo* in the ovine model for the first time. It was demonstrated an improvement in the histomorphometric results and osteointegration of the bone defects, demonstrating the improvement of the bone regeneration process.

This doctoral thesis also emphasizes the importance of including a multidisciplinary team in the development of new biomaterials and cell-based therapies, allowing a close participation of knowledge between the design of the biomaterials, the development of cellular therapies and the improvement of surgical techniques. Also, in this thesis it is evidenced the importance of the Veterinary Medicine in pre-clinical trials of human regenerative therapies and in clinical trials for application of these new therapeutically approaches in domestic animals, mainly companion animals and equine.

Keywords: Biomaterial, cells therapies, bone regeneration, synthetic bone graft, Bonelike®, stem cells, human mesenchymal stem cells from dental pulp, ovine experimental model, small animals, pre-clinical trials.

DECLARATION:

In compliance with the provisions of paragraph 1 of Article 34 of Decree-Law no. 74/2006, published in Diário da República, 1st series, nº 60 of 24th March 2006, and republished by Decree-Law no. 115 / 2013, published in Diário da República, 1st series, no. 151 of 7th August 2013, which proceeds to the third amendment of Decree-Law no. 74/2006 of 24th March 2006, the author declares that he is responsible for the design and execution of the work, as well as in the interpretation of the results and in the writing of the works, published and in publications, which form an integral part of this thesis and / or deriving therefrom.

Scientific Publications

Oral Presentations:

I. 3rd-5th December 2015 - V Luso-Brasilian Congress of Experimental Pathology at Escola Universitária Vasco da Gama, Coimbra, Portugal. "Bioglass-reinforced hydroxyapatite used for osteoregeneration: the ovine in vivo model" by LM Atayde, **JM Campos**, PP Cortez, JD Santos, AC Maurício (**Author of oral presentation**)
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III. 15th April 2016 - 1st Meeting of the Veterinary Sciences Doctoral Programme, ICBAS – University of Porto, Portugal. "Use of a mixture of hydroxyapatite and tricalcium phosphate as bone substitute in the regeneration of bone defects - clinical cases in companion animals." **JM Campos**, PO Pinto, AR Caseiro, C Mendonça, T Pereira, LM Atayde, JD Santos, AC Maurício (**Author of oral presentation**)
IV. 18nd February 2017 - Congress of Veterinary Hospital Montenegro at Europarque - Cultural and Congress Centre, Santa Maria da Feira, Portugal.

"Collection and clinical application of mesenchymal stem cells from dental pulp in regenerative medicine". (R Alvites, AR Caseiro, SS Pedrosa, **JM Campos**, IL Reis, A Varejão, AC Maurício) (**Co-author of oral presentation**)

V. 18nd February 2017 – Congress of Veterinary Hospital Montenegro at Europarque – Cultural and Congress Centre, Santa Maria da Feira, Portugal. "Treatment of non-union of long bones with macroporous synthetic substitute of hydroxyapatite and tricalcium phosphate – 3 clinical cases" by PO Pinto, **JM Campos**, AR Caseiro, JD Santos, LM Atayde, AC Maurício (**Co-author of oral presentation**)

VI. 24th January 2018 – Oral Communication at Departmental Seminar (lecture of 45 minutes about PhD project: "Development of biomaterials and cells therapies for bone regeneration" by **JM Campos**), chaired by Prof Matthew Allen at University of Cambridge, England, United Kingdom (**Author of oral presentation**)

VII. 26th February to 2nd March 2018 under ERAMUS+ Staff Mobility Teaching were presented 6 hours of lectures about this project at Poznán University of Life Science, Poland. «Veterinary Regenerative Medicine and Biomaterials – a set of grand challenges»; «The Potential Clinical Application of Mesenchymal Stem Cells from the Dental Pulp (DPSCs) for Bone Regeneration – Sheep as Surgical Experimental Model»; «Clinical Application of Stem Cells in Veterinary Medicine» by **JM Campos** (**Author of orals presentations**)

VIII. 28th September 2018 – 2st Meeting of the Veterinary Sciences Doctoral Programme, ICBAS – University of Porto, Portugal. "Use of Bonelike® as a synthetic bonegraft in small animals clinical trial" **JM Campos**, AC Sousa, PO Pinto, J Ribeiro, ML França, AR Caseiro, S Pedrosa, C Mendonça, LM Atayde, JD Santos, AL Luís, AC Maurício (**Author of oral presentation**)

IX. 8th October 2018 – Seminar in the scope of the course of Clinical Small Animals, at Escola Universitária Vasco da Gama, Coimbra, Portugal. "Veterinary Regenerative Medicine: Biomaterials and Cell Therapies by **JM Campos**" (**Author of oral presentation**)

Poster Presentations:

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II. 20th-22nd October 2016 – 51st Congreso Nacional AVEPA (Asociación de Veterinarios Españoles Especialistas en Pequeños Animales) and X Southern European Veterinarian Conference, Granada, Spain. "Use of a bioglass-reinforced hydroxyapatite synthetic bone substitute (BL®) for osteoregeneration of bone defects in small animal clinical cases" by **JM Campos**, PO Pinto, AR Caseiro, C Mendonça, T Pereira, LM Atayde, JD Santos, AC Maurício (**Author of poster presentation**)

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II. PO Pinto, LM Atayde, **JM Campos**, AR Caseiro, T Pereira, C Mendonça, JD Santos, AC Maurício (2017). Therapeutic strategies for bone regeneration: the importance of biomaterials testing in adequate animal models. *In* Advanced Materials Book Series, WILEY-Scrivener Publisher, USA. 21st February 2017.

III. AR Caseiro, R Alvites, SS Pedrosa, **JM Campos**, IL Reis, JD Santos, C Mendonça, LM Atayde and AC Maurício (2017). The Potential Clinical Application of Mesenchymal Stem Cells from the Dental Pulp (DPSCs) for Bone Regeneration. In eBook Frontiers in Stem Cell and Regenerative Medicine Research, Vol. 5 being published by Bentham eBooks. [eBook Reference No: 9781681084756-17-1151]

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II. LM Atayde, **JM Campos**, PP Cortez, JD Santos, AC Maurício (2016). Mesenchymal stem cells from dental pulp (DPSCs) and glass-reinforced hydroxyapatite synthetic bone - the sheep *in vivo* model for bone regeneration preclinical tests. Journal of the Portuguese Experimental Pathology Association - Experimental Pathology and Health Sciences 2016;8 (1): 33-34

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List of Abbreviations and Symbols

ALP	Alkaline phosphatase
AMSC	Adipose-derived stem cells
APDCs/SCAPs	Stem cells from human permanent teeth apical dental pulp
BCP	Biphasic calcium phosphate
BM-MSCs	Bone marrow derived macrophages
BMP-2	Bone morphogenetic protein 2
BMPs	Bone morphogenetic proteins
bMSCs	Bone marrow mesenchymal stem cells
BSP	Bone sialoprotein
Ca^{2+}	Calcium ion
CCIVV	Centro Clínico de Investigação Veterinária de Vairão
CD	Cluster of differentiation
CEMUP	Centro de Materiais da Universidade do Porto
CFU	Colony forming units
Ch	Chitosan
Coll	Collagen type
CS	Calcium sulphate
CXCR	Chemokine receptor
DBBM	Deproteinized bovine bone mineral
DCP	Dicalcium phosphate
Dex	Dexamethasone
DF-MSCs	Stem cells from human dental follicle
DMP-1	Dentin matrix acidic phosphoprotein 1
DMSO	Dimethyl sulfoxide
DPSCs	Dental pulp stem cells
DSPP	Dentin sialophosphoprotein
DTSCs	Deciduous teeth stem cells
ECM	Extracellular matrix
EDS	X-ray energy dispersion spectroscopy
EGF	Epidermal growth factor
EMA	European medicines agency
ESCs	Embryonic stem cells
EUVG	Escola universitária Vasco da Gama
FBS	Fetal bovine serum
FCS	Fetal calf serum

FDBA	Freeze-dried bone allograft
FEUP	Faculdade de Engenharia da Universidade do Porto
FMDUP	Faculdade de Medicina Dentária da Universidade do Porto
FTIR	Fourier transform infrared spectroscopy
G-CSF	Granulocyte-colony stimulating factor
GDPB	Granular deproteinized bovine bone
GMP	Good manufacturing practice
HA	Hydroxyapatite
hDPSCs	Human mesenchymal stem cells from dental pulp
HSCs	Hematopoietic stem cells
HT-PCL	Hyaluronic acid- tricalcium phosphate - polycaprolactone
HVUC	Hospital Veterinário Universitário de Coimbra
ICBAS	Instituto de Ciências Biomédicas Abel Salazar
IGF	Insulin-like growth factor
IGFBP-7	Insulin-like growth factor-binding protein 7
IL	Interleukin
ISCT	International Society for Cellular Therapy
ITS	Insulin-tranferrin-sodium selenite
KH2PO4	Potassium dihydrogen phosphate
LSCD	Limbal stem cell deficiency
MDPSCs	Mobilized dental pulp stem cells
MEPE	Matrix extracellular phosphoglycoprotein
MI	Myocardial infartation
MSCs	Mesenchymal stem cells
nHA	nano hydroxyapatite
nHAC/PLA	nano- hidroxiapatite/colagen/poli-L-lactic acid
NURR1	Nuclear receptor related 1 protein
O_2	Oxygen
OCN	Osteocalcin
OLIG2	Oligodendrocyte transcription factor
OM	Means of osteogenesis
ON	Osteonectin
OPN	Osteopotin
OSX	Osterix
P3	Passage 3
P4	Passage 4

PCL	Polycaprolactone
PDGF	Platelet derived growth factor
PDL	Periodontal ligament
PDLSCs	Stem cells from human Periodontal Ligament
PDO	Poly (p-dioxanone)
PLA	Poly Lactic acid
PLGA	Poly-lactic-glycolic acid
PMN	Polymorphonuclear Leukocytes
PRP	Platelet rich plasma
RA	Retinoic acid
RGD	Arginylglycylaspartic acid
rhBMP-2	Recombinant human bone morphogenetic protein 2
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SC	Schwann cells
SN	Study number
SEM	Scanning electron microscopy
SHEDs	Stem cells from human exfoliated deciduous teeth
SMF	Static magnetic field
SS	Stainless steel
STRO-1	Stromal cell precursor surface antigen
TCP	Tricalcium phosphate
TE	Tissue engineering
TeCP	Tetracalcium phosphate
TGF-β	Transforming growth factor beta
UCB	Human umbilical cord blood
UP	University of Porto
USA	United States of America
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VGFR	VEGF receptor
XRD	X-ray diffraction
α-SMA	Alfa smooth muscle actin
α-TCP	Alpha tricalcium phosphate
β-TCP	Beta tricalcium phosphate

Chapter 1 – General Introduction

Introduction

The population aging represents a global challenge for future end-of-life care [1]. Data from the United Nations Development Program and European Union shows significant gains in life expectancy. This increase represents a major achievement on the world community in advancing health wellbeing [1, 2].

With the aging population, there was an increase in diseases and bone fractures [3]. These emergent diseases include cardiovascular, neurological, musculoskeletal, orthopedic and several other malignancies. The ultimate goal of studying biomedical materials is to replace or create artificial organs and repair damaged organs and tissues of the human body in order to restore their physiological functions [4].

Bone substitutes are one of the most used medical devices, such as in orthopedic surgery and neurosurgery context [5]. Also craniofacial surgery is a concern because the bone loss by trauma, congenital deformities, tumors or infections are highly prevalent and often require complex reconstructive surgery [6]. More than two million bone grafts were performed annually worldwide, thus being the second most successful tissue transplantation following blood and blood derivate products [7]. The worldwide incidence of bone defect repair surgery is increasing and expected to double by 2020 [8].

Bone Tissue

Morphology and Bone Structure

This brief overview of the structure and development of the skeleton focuses primarily on bone and its cells [9]. Bone is a connective tissue containing cells, fibers and ground substance. It is a multifaceted organ consisting of several tissues that are the cortical (or compact) bone, the cancellous bone with the marrow compartment, and the periosteum. The periosteum cover external surfaces of most bones and is divided into two distinct layers – an outer fibrous and inner cellular layer (also called ‘cambium’) [10]. The cellular layer is positioned in direct contact with the bone surface

and is of particular interest as it contains osteoprogenitors cells and mesenchymal stem cells (MSCs) [11].

The bone is responsible for important functions in the living body, for instances providing a framework for attachment of muscles and other tissues; enabling body movements; providing protection of internal organs from injury; production of blood cells in the haematopoietic bone marrow; calcium homeostasis; acid/base buffering and body load-bearing and participating in biomechanical movement of the body [12, 13]. There are many functions in the body in which the bone participates, such as storing minerals, providing internal support, protecting vital organs, enabling movement, and providing attachment sites for muscles and tendons. Bone is unique because its collagen framework absorbs energy, while the mineral encased within the matrix allows bone to resist deformation [14].

Bone is a remarkably complex tissue. In what concerns to the composition and proprieties of specific connective tissues, the bone tissue is the main constituent of the skeleton, the most resistant and rigid tissue of the human body. Bone tissue is the main deposit of calcium and phosphate and contributes to mineral homeostasis thus releasing the ions in a controlled manner [7]. Unlike other tissues, the bone can regenerate and repair itself [15, 16]. In other circumstances, the body is unable to repair such defects in a considerable time, for example in pathological fractures or large and massive bone defects, bone healing and repair fail. Advances in biomaterials has allowed for the creation of medical devices to facilitate wound bone healing in both bone and soft tissues defects and injuries [17].

Large skeletal defects are often unable to heal spontaneously by the body's repair mechanisms and need to deal with large volumes of donor tissue with associated surgical procedures which has become a challenge. An experimental osseous injury inflicted to study bone repair mechanisms should be of dimensions to preclude spontaneous healing. Actually there is no one standard definition of a critical-sized defect. In general, a "critically-sized" defect is regarded as one that would not heal spontaneously despite surgical stabilization and requires further surgical intervention. Non-critical size defect ensures fast and reliable healing process, allowing the observation of the various stages of bone healing and biomaterial degradation [12, 18, 19].

In animal models used in preclinical studies, also exists controversy for bone defects. Some authors have suggested that a critical-sized defect is the smallest size intraosseous wound in a particular bone and species of animal that will not heal during the lifetime of the animal or a defect that shows less than 10% regeneration during the animal's lifetime [20]. A critical-sized bone defect will always require management of the defect, whereas a nonunion may or may not require management with a bone graft. There are numerous factors which affect bone defects and their "critical size" is dependent on the absolute versus relative size, whether there is circumferential loss of bone, anatomical location (diaphyseal/metaphyseal/articular), the soft tissue environment including injury to the periosteum and surrounding muscles, age, and presence of chronic diseases and other comorbidities [20]. Therefore, the non-regenerative threshold of bone was 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 animal" or as a defect which shows less than ten percent bony regeneration during the lifetime of the animal [21]. 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 bone. Results of various animal studies suggest that critical sized defects in sheep however, could be approximately three times the diameter of the corresponding diaphysis [22]. At non critical size defects, shorter implantation times allow to observe the early events of bone healing and bone regeneration. These shorter implantation periods allow to understand the biomaterial's performance, comparing controls (empty defects) with the created defects filled with a biomaterial. In order to study and understand the later stage of bone regeneration, as well as the biomaterial reabsorption and degradation, it is recommended to use longer implantation periods [21]. Nevertheless, a critical defect in a long bone cannot simply be defined by its size but may also be dependent on the species phylogenetic scale, anatomic defect location, associated soft tissue and biomechanical conditions in the affected limb as well as age, metabolic and systemic conditions, and related morbidities affecting defect healing [23].

Bone is a unique connective tissue with a hard extracellular collagen matrix that is impregnated with a mineral, manly hydroxyapatite. Collagen is the most abundant protein found in the body with a unique chemistry that makes it an ideal material for structural and haemostatic applications in both clinical and diagnostic

settings. Like all proteins, collagen is comprised of amino acids linked covalently through peptide or amide linkages. For example, its application in haemostatic medical devices in bleedings, has been described in several papers [17].

The skeleton forms the basic frame that supports the locomotor apparatus and from a mechanical point of view consists largely of a series of lever arms designed to counteract the force of gravity and to constrain and direct the forces of muscular contraction. Animals with extremely large body masses and with massive pillar-like limbs adapted to support their weight are said to be graviportal. Their typically columnar limbs help to resist bending and torsional loads generated by flexion and rotation of the limbs during locomotion. Bones vary greatly in shape among species, but molecular architecture is highly maintained [24-26].

Bone tissue in the adult skeleton is predominantly arranged in two architectural forms: Cortical also called compact bone or osteonal bone (around 80% of the total skeleton) is composed of longitudinally oriented, overlapping, cylindrical Haversian units that made up of central Haversian canal surrounded by concentrically arranged bone lamellae. Trabecular also called spongy bone, or cancellous bone (around 20% of the total skeleton) is located primarily in the metaphyseal regions or is projected into the medullary canal. It consists of thin and interconnected rods, plates, and struts called trabeculae [26, 27].

The inner and outer surfaces of a bone are covered by specialized connective tissues with an endosteal and periosteal membrane (**Figure 1**). Endosteal (medullary cavity) is a thin vascular membrane of connective tissue contains bone-lining cells, osteoprogenitors cells, and osteoblasts. Periosteum is a membrane that covers the outer surface of all bones and is rich in type I collagen, along with variety of proteoglycans and appreciable quantities of elastin [26]. The periosteum covers external surfaces of most bones and is divided into two distinct layers: an outer fibrous and inner cellular (or “osteogenic”) layer. The fibrous layer contains fibroblasts, while the osteogenic layer contains progenitor cells that develop into osteoblasts. These osteoblasts are responsible for increasing the width of a long bone and the overall size of the other bone types [14].

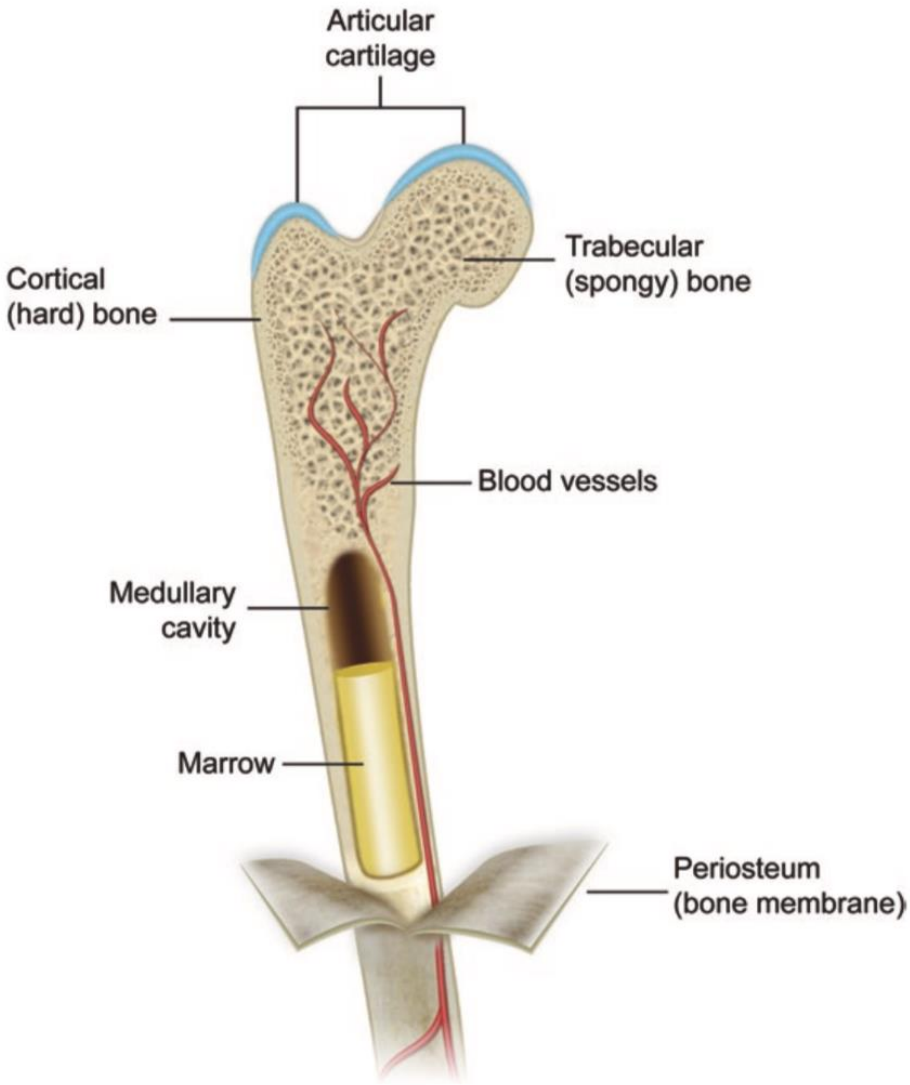


Figure 1: Long bones are composed of the cartilaginous joints, porous cancellous bone, the more compact, denser cortical bone, and the marrow cavity. The subperiosteal surface in long bones is covered by a thin fibrocellular highly vascularized and innervated membrane, the periosteum (Adapted from [28]).

These properties are due mainly to the remarkable hierarchical architecture, as is illustrated in **Figure 2** [29, 30].

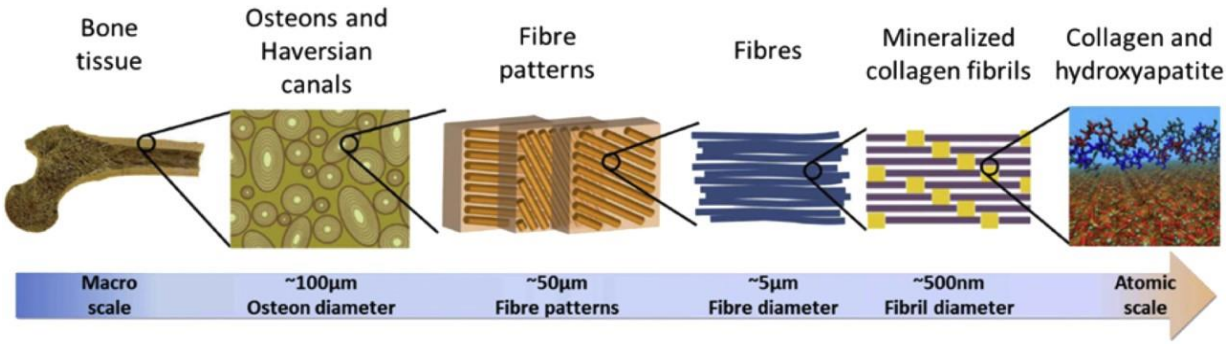


Figure 2- The hierarchical structure of bone ranging from microscale skeleton to nanoscale collagen and hydroxyapatite (Adapted from [31]).

Bone is a type of connective tissue composed of bone cells (osteoblasts, osteoclasts and osteocytes) and extracellular matrix, differentiating itself from the other connective tissues, once the matrix is mineralized (**Figure 3 and Figure 4**) [32].

Major cell types in bone include osteoblasts, osteocytes and osteoclasts. The activities of these cell types are both temporally and spatially coordinated during growth, remodelling, and repair of bone. This trio of cells therefore is termed the basic multicellular unit. This stimulation of osteoblast activity in response to resorption is termed “coupling” and it has long been of interest to understand how these two distinct cell types, on the same bone surface but at different times, could be linked so their activities are equal [33].

Skeletal mass is regulated by two key activities: bone removal (resorption) by haematopoietic lineage osteoclasts and bone matrix formation by mesenchymal lineage osteoblasts. During adult life, these activities occur sequentially on the same surface: a process termed as remodelling. Tiny packets of bone are removed by osteoclasts and replaced by new bone matrix produced by osteoblasts. This continual renewal process allows repair of mechanical imperfections and calcium homeostasis [33].

Osteoblast reside within the periosteal and endosteal membranes and constitute a heterogeneous population of cells that represent several distinct stages of lineage commitment, including osteogenic progenitors, pre-osteoblasts and osteoblasts. They have the responsibility for biosynthesis of the components of bone matrix and also express a wide variety of osteogenic growth factors and mediators. Their activity is regulated by a plethora of extracellular signals, including circulating hormones, autocrine and paracrine factors, cytokines, and chemokines [26].

Osteocytes are the most abundant cells in bone and are the major orchestrators of bone remodelling and mineral homeostasis. They possess a specialized cellular morphology and a unique molecular feature. Osteocytes are a stellate shape with numerous long, slender dendritic processes. The osteocyte cell body resides in the bone matrix of the lacuna and the dendritic processes extend within the canaliculi to adjacent osteocytes and other cells on the bone surface. Osteocytes form extensive intercellular network to sense and respond to environmental mechanical stimulus by the lacunar-canalicular system and gap junction. Osteocytes are long-lived bone cells. They can undergo apoptosis, which may have specific regulatory effects on osteoclastic bone resorption [34].

Osteocytes are terminally differentiated osteoblasts that have become encased in bone matrix during osteosynthesis. Within osteonal bone, osteocytes occupy individual lacunae within the concentric *lamalae* of Haversian system and intercommunicate with neighbouring osteocytes or osteoblasts via long filamentous cytoplasmic projections that extend through the matrix within minute fluid-filled *caniculi.* Osteoblast are the major orchestrators of osteoclastic recruitment, differentiation and activation through production of the monocyte colony-stimulating factor and the receptor activator of specific ligand. Thus, the effects of calciotropic hormones such as parathyroid hormone and parathyroid hormone-related peptide on the mobilization of calcium from bone are largely indirect, and are mediated by osteoblasts through the expression of receptor activator of specific ligand [26].

Based on the osteocyte location within the bone matrix and the cellular morphology, it is proposed that osteocytes potentially contribute to the control of bone remodelling by sensing mechanical stress and hormones. Furthermore, bone has been traditionally regarded as a part of the skeletal and locomotor system, but recent studies suggest that osteocytes regulate systemic biological functions based on the inseparable link between bone and other systems [35].

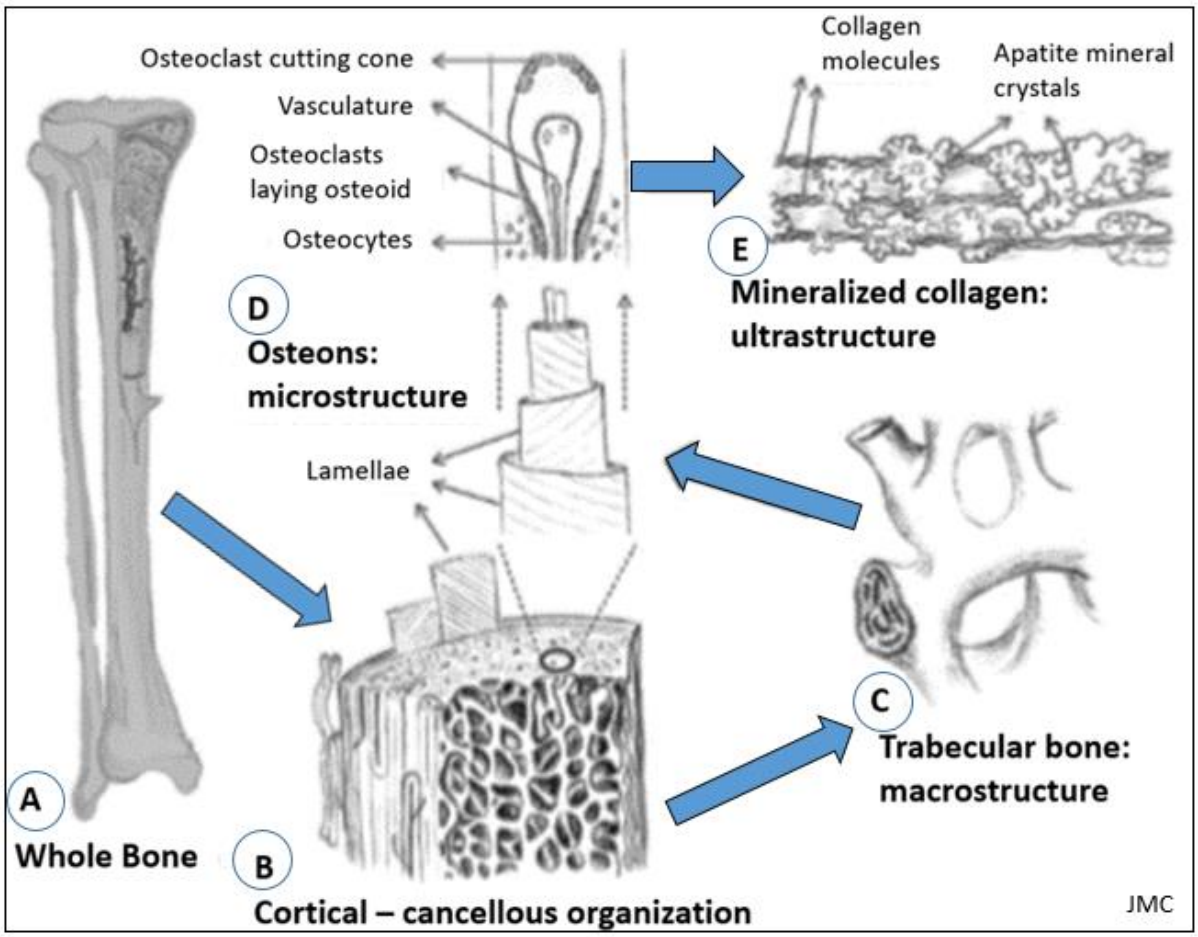


Figure 3 – The hierarchical organization of bone: (A) bones can be long or flat to meet physiological functional demands and are comprised of (B) a cortical and (C) a cancellous (trabecular) component. Based on formation and remodelling, (RD) the base functional unit of bone is an osteon with a central Haversian system for blood supply. At the ultrastructural level, (E) bone is a true composite of calcium phosphate mineralized on collagen fibrils (Adapted from [28]).

Osteoclasts are large multinucleated cells that derive from hematopoietic precursors of the monocytic lineage and are responsible for demineralization and proteolytic degradation of bone matrix. The formation and activity of osteoclasts are tightly regulated [26].

The endocortical surface of a bone faces the medullary canal and is lined by the endosteum, a single thin layer of bone lining cells (mature osteoblasts) and osteoblasts which form a membrane over endocortical and trabecular bone surfaces to enclose the bone marrow. The endosteum contains osteoprogenitor cells, but does not appear to contain either MSCs or hematopoietic stem cells (HSCs). However, a portion of HSCs can be found next to the endosteum suggesting reciprocal communication between cells within the endosteum and multipotent HSCs. The close

relationship between the cells forms a so called “stem-cell niche” whereby the cells of the endosteum physically support and influence stem cell activity [14].

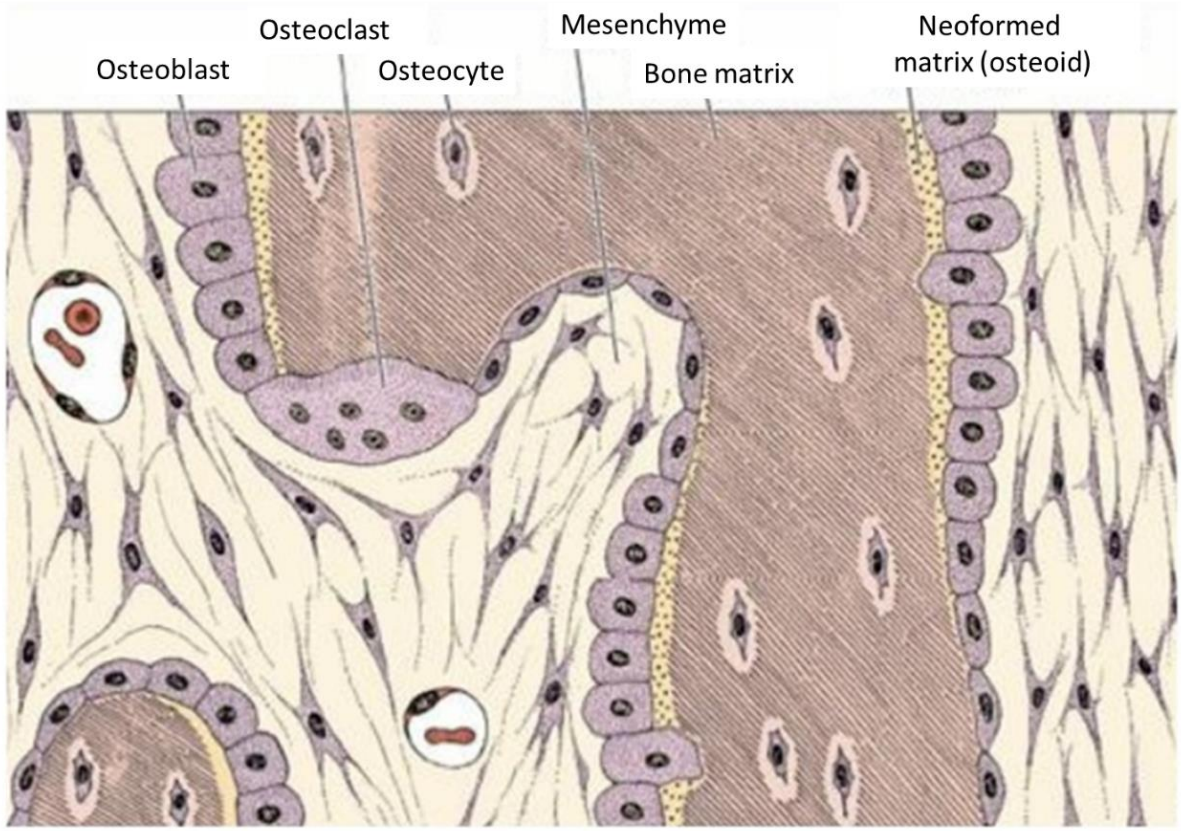


Figure 4 – Constituents of bone tissue (Adapted from [36]).

Living bone composition contains about 33% organic matrix and 67% inorganic compounds. The remainder consists of organic matrix with small quantities of cellular constituents. Mostly of collagen is the organic matrix (approximately 90%), glycoproteins, and proteoglycans. Gamma-carboxylate proteins, and other non-collagenous proteins, some of which are plasma derived (albumin, alpha-2-HS-glycoprotein) and sialoproteins. The remainder of inorganic matrix consists of ions such as phosphorus and calcium [26].

Bone mineral is primarily calcium hydroxyapatite, which has the molecular formula $(Ca_{10}[PO_4]_6[OH]_2$ and phosphorus, combined with small amounts of other minerals, including bicarbonate, magnesium, potassium, sodium, citrate ions and other trace impurities are also present in small amounts [27]. The crystals of HA are relatively small, being able to reach the size of 10 to 100 nm [37]. The junction of the HA with the collagen fibrils is responsible for the hardness and strength characteristics of the bone tissue [9].

Initial precipitation of apatite crystals takes place within specialised extracellular matrix vesicles produced by chondrocytes and osteoblast. Crystals are initially deposited within gap regions at the ends of individual collagen fibrils and then expand by both crystal growth and micro-aggregation in close association with fibrils. Collagen fibrils form the basic scaffold upon which mineralization occurs and the configuration of collagen fibrils within bone matrix can give rise to different forms of bone. In lamellar bone, fibrils are deposited in alternating orientation in a highly organized layered pattern, although in woven bone, fibrils are arranged in a more loose and disorganized manner [26].

The ultrastructure of bone contributes to its functional behaviour as well. Bone tissue is viscoelastic: the strength of bone depends upon the rate at which it is loaded. Bone is stronger when loaded rapidly than when load slowly. This tends to result from impact with high-loading-rate forces. In another illustration of metabolic optimization, viscoelasticity allows bone to be strong enough to sustain sudden impact, such as falling, without so much to impair normal locomotion. A cortical bone property related to its ultrastructure is anisotropy because the mechanical proprieties of bone strongly depend on the direction of loading, long bones generally better resist forces placed along the long axis than those across the axis. This makes evolutionary sense as well in that long bones are usually oriented perpendicular to the forces of gravity [38].

The bone tissue presents distinct characteristics, depending on its state of maturation. Thus, it can be divided into two types of tissue: i) the primary or immature bone tissue that is predominantly found in the foetus. It also appears in growing bones or during the repair of fractures and in some pathological conditions in adults [9]; ii) the secondary bone tissue exists in places where stresses are exerted. It replaces the primary bone during the process of growth or bone regeneration. This is divided into two types of tissue: the predominant lamellar bone, and the fibrous or fascicular bone [9].

The mechanical properties of the bone reconcile a high stiffness and elasticity not yet found in synthetic materials. In the development of implants for bone regeneration must be considered the characteristics that can affect the growth of this

tissue in the affected sites, so it is important to know the mechanism of bone growth [32].

Microscopic bone structure

Microscopic visualization of both cortical and trabecular bone reveals tissue that is either woven or lamellar in structure. Woven bone has a disorganized collagen fibril arrangement and is not typical in the adult skeleton, except in pathological conditions (such as Paget's disease and osteosarcoma) or following injury (such as fracture). The disorganization of woven bone results from the speed at which it forms, which precludes the orderly deposition of collagen fibrils. The net result is bone tissue that possesses enhanced flexibility at the cost of stiffness. This is valuable following injury as rapid, early formation of woven bone enhances early restoration of skeletal mechanical integrity prior to its replacement by lamellar bone [14].

Lamellar bone is characterized by the organized arrangement of collagen fibres into layers or lamellae. This arrangement gives lamellar bone greater stiffness compared to the disorganized structure of woven bone. Lamellae in cortical bone form osteons or bone structural units, which consist of a central canal enveloped in concentric lamellae of bone tissue. Outer lamellae form first along the boundary of the osteon known as the cement line, with each successive lamella being laid concentrically inside the preceding one. In trabecular bone, lamellae are stacked into saucer-shaped bone packets that are separated by cement lines. The first lamellae are formed towards the centre of the trabeculae with each successive lamella being stacked in parallel layers towards the bone surface. Uniformly spaced throughout lamellae are lenticular cavities called lacunae from which branching *canaliculae* radiate in all directions. The c*analiculae* penetrate the lamellae of the interstitial substance to anastomose with *canaliculae* of neighbouring lacunae to form a continuous network of interconnecting cavities [14].

Bone Healing and Remodelling

The repair of bone fractures is a regenerative process that recapitulates many of the biological events of embryonic skeletal development. The initial fracture causes a local disruption of the vascular network which leads to haematoma formation, closely followed by the acute inflammatory phase. The following fracture healing

process largely recapitulates the process of long bone development in the embryo, including: i) migration of skeletal precursors to the site of skeletogenisis; ii) cellular condensation; iii) differentiation towards chondrocytes and/or osteoblasts [39].

The biological processes involved in bone regeneration require three critical elements as follows: an osteogenic potential that is capable of directly providing cells to the newly forming bone, osteoinductive factors that are able to cause the osteoblastic differentiation of osteoprogenitor stem cells, and osteoconductive scaffold that facilitates neovascularization and supports the ingrowth of bone. The ideal bone graft material possesses all of these three properties along with an optimal biological reaction and without a risk of transmission of diseases [40].

Bone has a unique ability to self-regenerate and remodel to a certain extent throughout life, without scarring since it is a highly vascularized, living and dynamic tissue [41]. Most fractured bones will heal without surgical intervention or even external coaptation. However, the healed bone ultimately may not function adequately because of limb alignment abnormalities (*malunion*), soft tissue contracture, or secondary osteoarthritis in the case of articular fracture. Therefore, the main goal of fracture repair should be to attain an end functional result equivalent to pre-fracture function. Understanding the varied courses of bone healing and how surgical intervention affects these courses is essential for choosing the appropriate treatment method for a particular fractured bone [38].

Bone is constantly renewed by the balanced action of bone formation and bone resorption both of which mainly occur at the bone surface. This restructuring process called "bone remodelling" is important not only for normal bone mass and strength, but also for mineral homeostasis. An imbalance of bone remodelling is often linked to various bone diseases and pathologies. During bone remodelling, resorption by osteoclasts precedes bone formation by osteoblasts. Based on the osteocyte location within the bone matrix and the cellular morphology, it is proposed that osteocytes potentially contribute to the controls of bone remodelling by sensing mechanical stress and hormones [42].

The bone originates mostly in pluripotent stem cells and multipotent mesenchymal cells during embryonic development. There is also the formation of bone tissue during growth, in the remodelling process, in the treatment of fractures

and after the implantation of osteoinductive matrices [11]. In the process of osteogenesis, which consists of the formation of bone tissue, pluripotent stem cells and multipotent / mesenchymal cells allow a cascade of differentiation steps, in this order: pluripotent stem cells and multipotent / mesenchymal cells, immature osteoprogenitor cells, mature osteoprogenitor cells, pre-osteoblast, mature osteoblast, and osteocyte [43, 44].

Most remodelling occurs on cortical or trabecular surfaces however a similar remodelling process can also occur within the Haversian systems of cortical bone. Osteoclastic precursors are recruited to areas of bone damage via Haversian vasculature. Differentiation and activation of these cells leads to a formation of a cluster of osteoclasts that can migrate along Haversian tract, creating a longitudinal resorption tunnel within the compact bone. This group of osteoclast forms the leading front of a cutting cone and is followed by populations of osteoblasts that deposit new concentric lamellae of osteonal bone in the path of the cone. Formation of cutting cones and direct cortical remodelling occurs during primary fracture healing, which itself can be considered a form of bone healing by direct remodelling [38].

Some fundamental principles of tissue engineering must be respected [62]. For example, in bone grafts, regeneration of bone tissue requires the presence of cells capable of forming new tissue and factors that stimulate their phenotypic differentiation in osteoblasts [61].

The later the differentiation step, the lower the capacity for self-regeneration and cell proliferation. The duration of the formation phase of the bone tissue is distinct between animal species. While, for example, it can take one to two weeks in the man, the dog lasts a week and in the rabbit a few days. Filling a bone cavity in the rabbit takes four to five weeks, in the dog ten weeks and in the man about thirteen weeks. That is, the duration of the bone remodelling cycle usually increases in direct proportion to the size of the animal and its metabolism. Relating the duration of the bone remodelling cycle in the various animal species, it is possible to calculate the time required in humans to obtain a behaviour similar to that of the bone tissue in the animals after the implantation of a similar biomaterial [45].

Healing of bone fractures

Bone fracture healing is an orchestrated complex regenerative process that involves a crucial number of progenitor cells such as inflammatory, endothelial and hematopoietic cells. The cellular and molecular events are strictly regulated during the healing cascade which includes the initial inflammatory phase, hematoma formation and progenitor cell recruitment [39]. So, unlike other tissue types, bone healing is the repetition of ontological events that occur during the embryonic development of the skeleton, which allows the damaged organ to completely restore its composition, structure and function [46].

Efficient dynamic bone balance prevents bone fracture, with the exception of the appearance of a load that exceeds bone strength or damage gradually accumulated under cyclic loading [47]. Thus, bone fracture or placement of an implant results in loss of continuity of bone tissue at the site of injury and is associated with injury to surrounding tissues. In both cases (fracture or implant), healing is a complex process involving multiple cellular and extracellular events and the ligaments, muscles or blood vessels are damaged resulting in hemorrhage [48, 49].

Hemorrhage in these cases results in the activation of the coagulation cascade and in the final formation of a blood clot and hematoma formation [48, 49]. After the hematoma appearance, an acute inflammatory response occurs. Inflammatory cells (macrophages, monocytes, lymphocytes and polymorphonuclear cells), fibroblasts and endothelial cells are incorporated into the bone. Necrotic tissue and foreign bodies are removed by leukocytes. Then there is the formation of granulation tissue, angiogenesis and migration of osteogenic population [48–50]. These cells will proliferate and differentiate and invade the bone chamber or spread over the surface of the bone (or implant) [48]. Much of the normal healing process is driven by growth factors, inflammatory mediators and cytokines [48–51].

In fracture healing, skeletal and immune system are closely interacting through common cell precursors and molecular mediators. It is thought that the initial inflammatory reaction, which involves migration of macrophages into the fracture area, has a major impact on the long term outcome of bone repair. Interestingly, macrophages reside during all stages of fracture healing [52].

Bone repair can be defined in two categories:

i) Primary bone healing occurs mainly when the fracture interval is less than 0.1 mm and the fracture site is stabilized. It is assumed that the bone gap in this process is filled directly by continuous ossification and subsequently the remodeling of Haversian [53, 54], with absence of cartilaginous or connective tissue [54, 55].

ii) Secondary bone healing is the most common form and occurs when the fracture limits are less than twice the diameter of the injured bone [54]. Blood coagulation, inflammatory response, formation of fibrocartilage callus, intramembranous and endochondral ossification, and bone remodeling are involved in the healing of secondary bone fracture [56, 57].

The biological events and activities, as well as the cells involved in the healing of bone fractures at different stages, are illustrated in **Figure 5** [58].

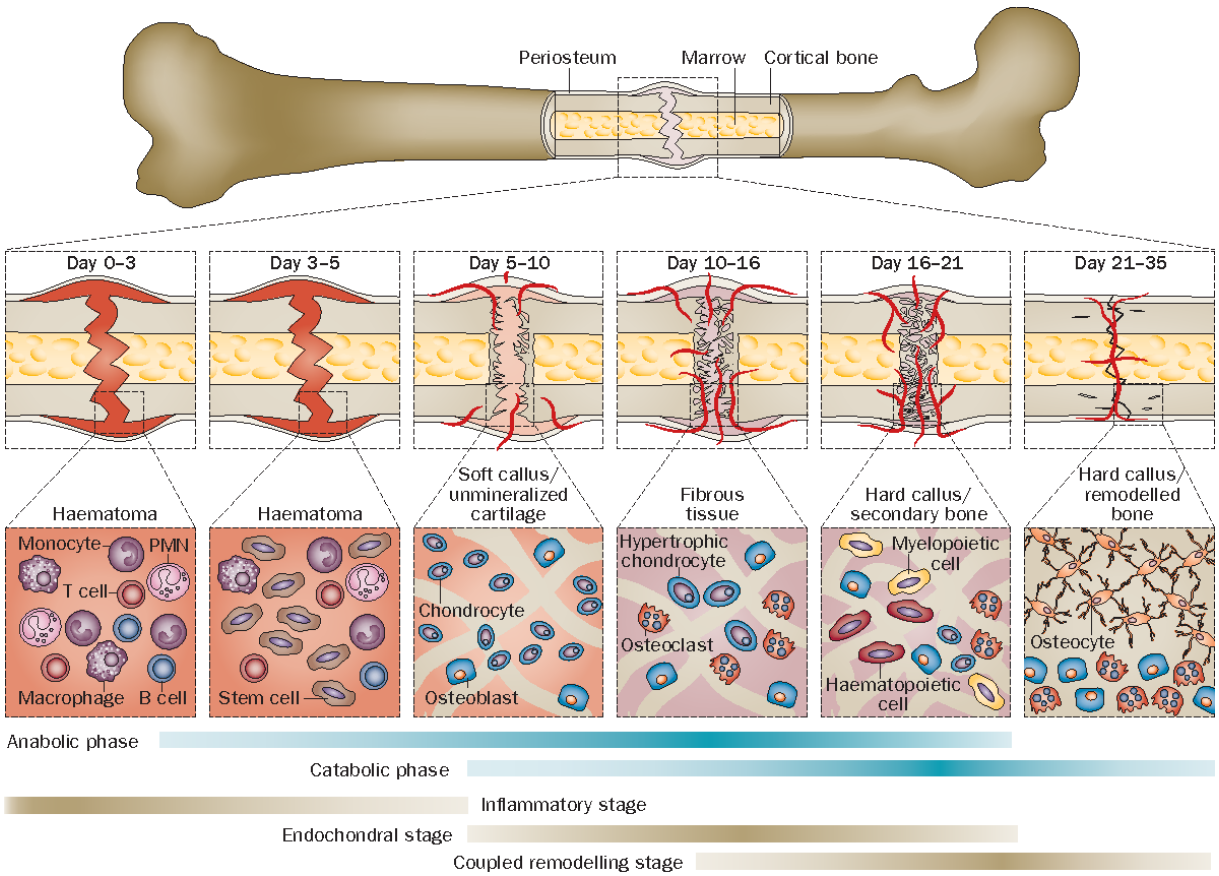


Figure 5 – Illustration of a typical healing process of fractures, biological events and cellular activities in different phases. The primary metabolic phases (blue bars) overlap the biological phases (brown bars). Abbreviations: PMN, polymorphonuclear leukocytes (Adapted from [58]).

Segmental bone defects or critical-size defects are an extreme condition in bone healing, which can be caused by trauma, disease, developmental problems,

revision surgery, tumor resection or osteomyelitis [7]. The extensive loss of bone, in these cases, directly affects revascularization and tissue differentiation and may eventually lead to spontaneous bone fracture, which does not heal without interventions. Critical size bone defects are defined as the least defect in a specific bone of a species that does not heal spontaneously during the useful life of the species [59, 60].

These events may influence patients' quality of life due to long-term and postoperative treatment costs and also to major surgical, socioeconomic and research challenges [7].

Biomaterials

Nowadays, biomaterial is defined as a medical device suitable for inclusion in systems which augment or replace the function of bodily tissues or organs and can interact with biological systems [61]. In other words biomaterial can be defined as any kind of substance used to treat, replace or enhance any live tissue, interacting with it without causing any adverse reactions in the body and its components [62].

Since biomaterials will react with the surrounding tissues after their implantation, cytocompatibility and histocompatibility are very important issues to be tested before pre-clinical trials. Thus the main requirement of a biomaterial is its biocompatibility capacity, property in which the foreign material provides a response when implanted in living tissue without damaging all the surrounding tissue. Biocompatibility can be defined as the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy [63-65].

The ideal biomaterial should comply with some important criteria, namely possess appropriate mechanical properties to support new bone formation, interconnected porosity to allow cell migration and exchange of nutrients, to have antimicrobial properties in order to eliminate bacterial infection in orthopedic implants and cytocompatibility to allow further cell proliferation [66].

The choice of the bone graft is dependent on many variables, including individual patient factors, tissue diagnosis, size and location of the lesion, and risk of pathologic fracture. Options include autograft, demineralized allograft, bone cement, and various synthetic grafting materials. Freeze-dried allograft cancellous bone chips are among the most commonly used grafting options for the treatment of closed metaphyseal bone defects and offers low complication and reoperation rates. Freeze-drying and irradiation of allograft bone chips to exclude the possibility of disease transmission and to reduce its immunogenicity greatly diminishes its osteoinductive properties [67]. There are other attributes that must be considered such as its ability to be adjusted to a particular function to undergo sterilization and the possibility of being manufactured at minimum cost and maximum reliability [68].

In order to provide a better bone regeneration using bone grafting, it should be use a bone graft reabsorbed, that can confer resistance similar to cortical bone, have osteoconductive and osteoinduction properties, be easy to manipulate, sterilizable and not cause adverse reactions. Unfortunately, still does not exist a perfect biomaterial that can cover all these features [69].

Classification

In general, biomaterials can be classified according to their chemical composition and biological behavior (**Figure 6**). The first separates the biomaterials into metals and metal alloys, ceramics, polymers and composites [70]. The classification according to the biological behavior is based on the response of the host tissue [65, 71]:

1. **Bioinert**: the material is not caused by a foreign body in the organism, that is, it must not be toxic, carcinogenic, antigenic (it must not be identified by the organism as a foreign body), mutagenic (it must not induce genetic mutations); It is in direct contact with the tissue receptor. Corresponds to the type of biomaterial that triggers a minimal reaction with the surrounding tissue, such as stainless-steel, titanium, aluminum oxide and zirconia; one such situations, non-adherent, thin fibrous capsule is formed around the implant, without a direct link (mineralized) between the bone and the biomaterial. In opposition, a toxic material causes an adverse

reaction in the surrounding tissue of the implant, taunting cellular dead [71].

2. **Bioreabsorbable**: the biomaterial is slowly degraded and gradually replaced by the tissue where it is implanted, i.e., it means that dissolves (absorbs) over time and it is replaced by new bone tissue, such as the tricalcium phosphates. The type of bioactive material that interacts with the surrounding tissue, existing ion exchange reactions with the surrounding fluid and the formation of carbonated apatites, forming strong and direct connections between the bone and the biomaterial with a strong single interface, such as TCP and Calcium Sulphate (CS) [71].

3. **Biotolerated**: the biomaterial is moderately accepted by the receptor tissue and is usually surrounded by a fibrous capsule. Examples of this biomaterial are stainless steel (SS), chromium-cobalt (Cr-Co) alloys and polymethylmethacrylate (PMMA) [71].

4. **Bioactive**: the biomaterial forms a direct bond with living tissue, since they usually contain calcium and / or phosphorus ions (in the case of bone substitutes) that will establish a chemical bridge with the surrounding bone. Examples of this biomaterial are HA and bioactive glasses [71]. The bioactive type of material is one that interacts with the surrounding tissue, such as hydroxyapatite, promoting ion exchange reactions with the surrounding fluid and the formation of carbonated apatites, forming strong and direct connections between the bone and the biomaterial with a strong single interface [68, 72].

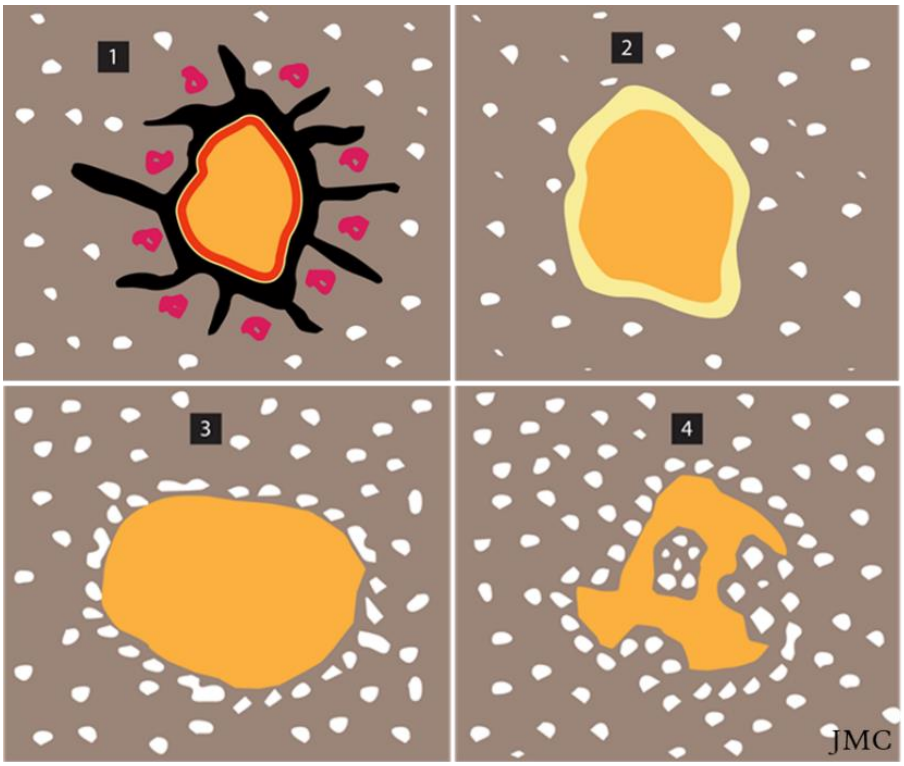


Figure 6 - The representation of a response of a biomaterial in a living organism. 1-Toxic; 2-Bioinert; 3-Bioactive; 4-Bioreasorbable (Adapted from [73]).

A part of the most studied bioactive biomaterials are ceramics. Bioactive ceramics including HA, β-tricalcium phosphate (β-TCP), biphasic calcium phosphate (BCP), bioactive glasses and calcium silicates have been widely used for bone tissue engineering applications [74]. These materials can be easily fabricated into scaffolds with pore structure matching that of cancellous bone and due to their compositional and surface characteristics, can form a direct chemical bond with bone which facilitates interactions with osteoprogenitor cells to encourage bone formation [45].

In bone tissue engineering, a scaffolding material is used either to induce formation of bone from the surrounding tissue or to act as a carrier or template for implanted bone cells or other agents. To serve as a scaffold, the material must be biocompatible, osteoconductive, and osteointegrative, and have enough mechanical strength to provide structural support during the bone growth and remodeling [75].

The key challenge that remains to be solved in the field of bone tissue engineering is to create purely synth etic scaffolds which have the combined properties of:

i) High porosity and pore interconnectivity to facilitate bone ingrowth, vascularization and nutrient/waste transport;

ii) Mechanical properties matching native bone tissue to provide mechanical stability at load-bearing defect sites;

iii) The ability to promote biological interactions with bone-related cells to actively induce bone formation [76].

Bone tissue engineering aims to combine and deliver the cellular (cells), extracellular (scaffolds) and/or molecular elements (cues/signals, growth factors) involved in physiological regenerative processes, for therapeutic applications. This usually involves harvesting of osteogenic cells from an autologous source (e.g. bone marrow, adipose tissue, umbilical cord blood and matrix, dental pulp, among other sources), the cells *in vitro* culture for expansion and combination with an appropriate carrier scaffold for posterior *in vivo* implantation [77]

Biomaterials interacts with biological systems in order to evaluate, treat, increase or replace tissues, organs or functions of the organism [61, 78, 79]. There are many biomaterials applied in the human body (**Figure 7**) [80].

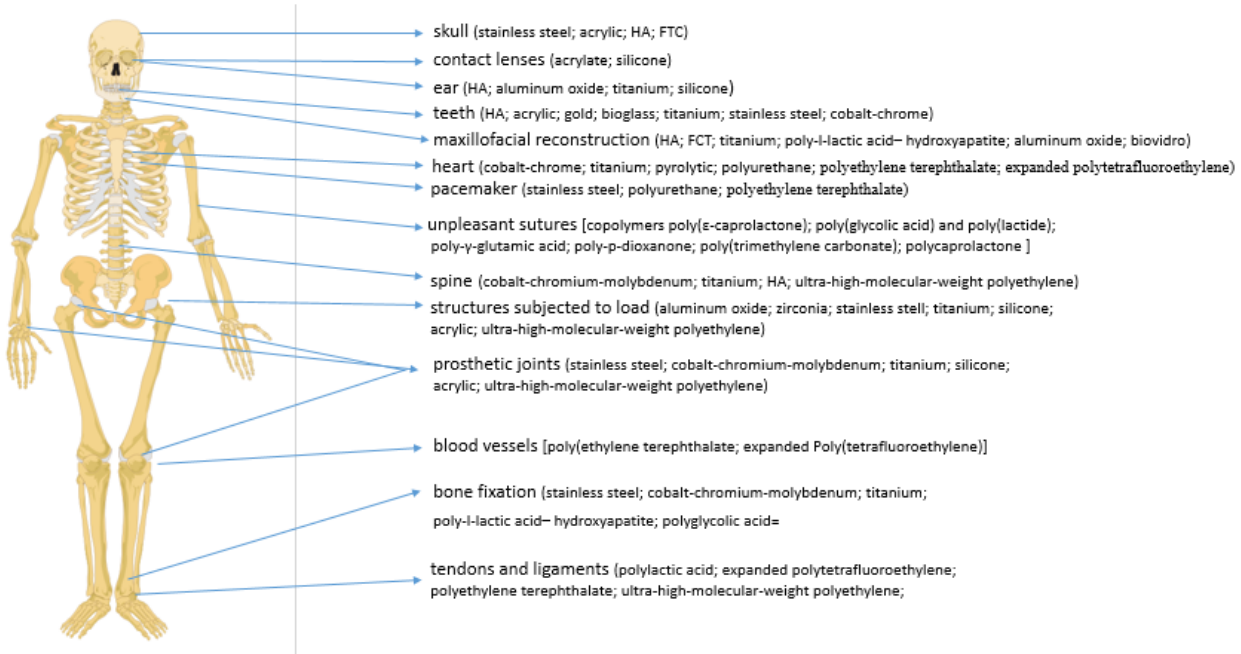


Figure 7 – Examples of biomaterials applied in the human body (Adapted from [81]).

Bone grafts

Over 2.2 million bone graft procedures are performed annually worldwide for the repair of bone defects in orthopedics, neurosurgery and, dentistry but also, in plastic and reconstructive surgery in humans [76]. The repair of organic functionality and reconstruction of bone defects is a major clinical problem and traditional reconstruction by grafting is time consuming, with significant morbidity for the host area and sometimes results in limited bone quality and quantity [4, 6]. This justifies the reason why currently bone continues to be the second most transplanted tissue in the world, right after blood and its derivates [5, 7, 82]. Nevertheless, the development of a bone graft with optimal properties remains a clinical challenge [5]. Researchers, are looking for new and better products and methods to enhance the body's own mechanism to produce a rapid healing process [17].

Finding the ideal bone substitute has been the focus of research in the field of bone regeneration. Bone tissue engineering, which uses seed cells, biologically active factors and scaffold composite materials to restore the structure and function of bone, provides an ideal solution for the clinical bone defect problem [4]. The current market for bone graft substitutes is dominated by bioactive ceramics composed of calcium phosphates and bioactive glasses [83].

Currently, bone grafts are used to restore skeletal integrity, to treat bone defects, delayed union, non-union and fusion in orthopedic-neurosurgery [84]. The most used graft remains the autograft, which has the ability to provide osteoinductive growth factors and does not induce immunological reactions. It also provides a scaffold for osteoprogenitor cells and viable cells, while avoiding disease transmission. Still is considered to be the technique of choice in almost bone fusions, for small gaps and in critical defects given its properties of osteoconduction, osteogenic and osteoinduction [85].

Bone grafting is involved in virtually every procedure in reconstructive orthopedic surgery. Although autologous bone grafts have excellent biologic and mechanical properties, considerable donor site morbidity and the limited volume available must be taken into consideration. Currently, there are no heterologous or synthetic bone substitutes available that have superior biologic or mechanical properties [86].

Bone regeneration and healing is an area of extensive research providing an ever-expanding set of not only therapeutic solutions for surgeons but also diagnostic tools. Multiple factors such as an ideal graft, the appropriate biochemical and mechanical wound environment, and viable cell populations are essential components in promoting healing. While bony tissue performs many functions, critical is mechanical strength, followed closely by structure. Many tools are available to evaluate bone quality in terms of quantity, structure, and strength [87, 88].

Bone tissue regeneration is a dynamic process that relies on mutiples growth factors and cell types, however this ability is not adequate in some large defects and it is therefore necessary to find alternatives to promote bone healing [89].

The biologic mechanisms that dictate bone grafting are based on the bone formation processes of osteogenesis, osteoinduction, osteoconductive, osteointegration and, osteopromotion [90, 91].

i) **Osteogenesis** is the formation of new bone by the graft itself. The grafts need to contain viable osteoblasts that deposit osteoid, or stem cells that are induced by osteogenic precursor cells and differentiate into osteoblast within a graft. Implies the existence of living osteoprogenitor cells in the graft, which can proliferate and differentiate into osteoblasts and osteocytes [86].

ii) **Osteoinduction** is the stimulation and activation of adjacent host mesenchymal stem cells (MSCs) to differentiate into cells involved in bone growth, including osteoblasts and osteoclasts. This is caused by growth factors released by the bone graft. There is ability of the graft to stimulate undifferentiated MSCs, near the implant tissue, to differentiate into osteoblasts and begin to grow new bone tissue. The osteoinductive properties of a graft depend on the availability of growth factors. In fresh autologous grafts, several growth factors are detectable [86, 92].

iii) **Osteoconductive** is the provision of a scaffold matrix that allows healing tissue to grow across it. It refers to the capacity to induce the formation of new bone through the graft and this includes vascular, fibrous, and cartilaginous and bone tissue phases. Osteoconductive

properties depend on the three-dimensional structure of the graft and determine the velocity of osteointegration. This is well illustrated when comparing the osteointegration between dense cortical grafts and highly porous cancellous grafts: the cancellous graft is incorporated much faster [93, 94].

iv) **Osteointegration** refers to the ability to interconnect with original graft bone, it means is the process by which the graft material is integrated into surrounding host bone [95, 96] [27].

v) **Osteopromotion** is the enhancement of bone formation without being directly osteoinductive or osteoconductive. For example, enamel matrix derivate alone has no osteoinductive proprieties, but it can enhance the osteoinductive effect of demineralized bone matrix. Alternatively, creating a vascular and oxygen-rich environment allows bone healing to proceed [97].

Sum up, bone grafting is the transplantation or implantation of bone or a bone substitute into a site to replace missing bone or to enhance bone healing and there are several indications for grafting, such as complex fractures, fractures with poor biological healing potential, filling of bone defects, arthrodesis, intervertebral fusion and treatment of delayed or non-union fractures. In small animal orthopedics, there are a huge variability of bone injuries resulting in different biologic needs and biomechanical forces which should be evaluated by the surgeon. The selected material should meet those requirements, while minimizing costs and complications [98]. Currently, both biological and synthetic grafts have been used for bone regeneration. Biological grafts include autografts, allografts, and xenografts. Autografts consist of removing bone tissue and grafting it into the same individual; allografts consist of bone tissue donated by another individual or procured from cadavers, and xenografts are bone tissue obtained from other species. Nowadays, synthetic grafts have emerged as a viable alternative to biological grafts.

Autografts

Autografts are autogenous bone grafts or autologous bone grafts and are harvested from the recipient´s own body. It can be defined as piece of bone collected for grafting purposes from a donor site within the same individual [97]. Autografts are typically comprised of cancellous bone and/or cortical bone [17]. Cancellous bone grafts provide minimal structural integrity. Bone strength increases as the implant incorporates surrounding cells and new bone is formed. For cortical bone, the graft initially provides some structural strength. However, as the graft is incorporated by the host bone, nonviable bone is removed by resorption significantly, reducing the strength of the graft [17]. Insufficient blood supply, infection of the bone or the surrounding tissues and systemic diseases can negatively influence bone healing, resulting in delayed unions or non-unions [15]. Reconstruction of large bone defects and nonunion fractures of long bones are still challenging and the current gold standard surgical techniques involve autologous bone blocks harvested from the iliac crest, scapula or radius, and transplanted at the recipient site [99, 100].

Over the past 30 years, a wide range of synthetic scaffolds have been developed as bone graft substitutes for overcoming the issues associated with autograft and allograft transplantation. The use of autograft bone may result in severe patient pain and other complications at the harvest site and there are limitations to the amount of autograft bone that can be harvested from the patient [7]. However, the use of autografts is associated with longer anesthetic times, limited availability of bone graft that can be collected from the patient and the viability of the cells after harvesting, pain, intra-operative blood loss, stress fracture, risk of local infection and predisposition to failure. Despite of its particular features, there is an increasing use of synthetic bone because of the high morbidity of the harvest and limitations of the volume of autografts [17]. Limitations and risks associated to this type of autografts are widely described. It includes the transmission of immunological reaction, prionic diseases and virus contamination. Another inconvenient is that it requires a second surgical intervention for its collection and the amount of tissue available is limited [101].

Autografts are the most known form of grafting bone and considered an excellent bone graft in terms of osteoconductivity because they have the ability to provide osteoinductive growth factors. They also have an osteogenicity and osteoinduction features with histocompatibility minimal adverse immunological reactions. They provides a scaffold for osteoprogenitor cells and viable cells, while

avoiding disease transmission [17]. So, for these reasons, this is the most commonly used form of bone graft. Cancellous bone, cortical bone, or a mix of both, are used. The different types of autografts have variable properties associated with the structural anatomy of the harvested site. Cancellous bone has greater cellular activity and diversity than cortical bone, while autologous cortical grafts have enhanced mechanical properties [102]. Autografts are the current strategies for filling bone defects and subsequent repair, but each type has drawbacks [103].

Different types of autologous bone grafts have variable properties associated with structural anatomy. Cancellous bone has greater cellular diversity and activity than cortical bone, whereas cortical grafts have enhanced mechanical properties.

Overall, cancellous bone is eight times as metabolically active as cortical bone and cortical bone is four times as dense as cancellous bone [86, 94].

Autografts bone grafts have different types, include:

i) Cancellous autografts;
ii) Cortical-cancellous autografts;
iii) Non-vascularized autografts;
iv) Vascularized cortical bone graft.

i) Cancellous Autografts

The trabecular structure of cancellous bone results in a large surface area and don´t involve any kind of complex process, it is immediately ready for grafting. This allows for a high number of cellular components (mesenchymal stem cells, immature and mature osteoblasts) to be incorporated and explains its excellent osteogenic and osteoinductive capabilities [96, 104, 105].

Graft is harvested from long bones, introducing a curette into the metaphysis of long bones from where cancellous bone is then retrieved. Cancellous bone has excellent osteoconductive proprieties, but provides no structural support. Cytokines and growth factores within the graft contribute to osteoinduction, including transforming growth factor beta 1 (TGF-ß1), bone morphogenic proteins (BMPs), epidermal growth factor, platelet-derived growth factor, fibroblast growth factor and vascular endothelial growth factor. Osteogenesis mechanisms have the contribution of osteoblasts on the surface of the graft

can survive from transplantation. In addition, the trabecular structure allows for easy revascularization and rapid incorporation at the host site [105].

In turn, one of the limitations of cancellous bone is its lack of initial mechanical strength. Also, the formation of new bone on a necrotic cellular structure weakens the construct within the first weeks. As a result of the excellent biologic capacity to induce the production of new bone, increased stability is then achieved within months once the graft is incorporated. Vascularization of cancellous bone grafts begins within 2 days after implantation and is accompanied by infiltration of the marrow spaces by mesenchymal stem cells [105].

The early stage of osteointegration with revascularization is followed by graft remodeling with active bone formation and resorption of necrotic bone after 4 weeks. Histologically, osteoblasts can be found to line the trabecular scaffolds and deposit a seam of osteoid.24 Remodeling takes several months. Cancellous bone is more osteogenic than cortical bone as it has a low density porous structure that allows diffusion of nutrients and growth factors, inducing MSCs to differentiate and propagate [86, 93].

ii) Cortical-cancellous autograft

This kind of grafts can be harvested in different ways and is a mix of cortical and cancellous bone. Initially cortical and then cancellous bone is cut but the final result is a mix of finely morselized bone graft. An example would be application of a reamer to the lateral iliac wing [97].

iii) Non-vascularized cortical autograft

This is a section of resected cortical bone that are harvested and grafted. Cortical bone grafts have a more limited biologic profile as compared with cancellous grafts. Once the graft has re-vascularized osteointegration should occur, but this may be a very slow process taking up to one year [97].

At the time of transplantation, the grafted section of bone is avascular and could be a dramatic limitation because potentially create an infection. For instant, the use of non-vascularized ulnar and rib grafts into a mandibular non-union defect in dogs has been reported. Cortical bone has fewer osteoblasts and osteocytes, fewer growth factors, and less surface area per unit weight, the structure of which constitutes a barrier to vascular ingrowth and remodeling [106].

iv) Vascularized cortical bone grafts

Vascularized grafts have favorable biologic attributes as compared with standard autologous non-vascularized cortical grafts. It is an intricate procedure that consist in the transplantation of a bone segment including its vascular supply. For example, the transplantation of a section of ulna, vascularized by preservation of the caudal interosseous artery and vein, into adjacent radial bone tumor [107].

The use of homogenous whole cortical grafts is not an established procedure however; these grafts have been used in repairing multiple fractures of the femur where replacement of bone fragments was either not possible or would not lend any support to the fracture area. Whole cortical grafts could also be used in compound fractures with loss of bone substance, as a means of lengthening long bones, to shorten the surgical procedures, and in treating osteomyelitis and bone tumors [108].

It has been suggested that if adequate vascular anastomosis and graft stability are achieved, more than 90% of the osteocytes may survive the transplantation [109].

The main advantage is that the grafted bone retains its vascular supply and biological features. New bone formation by graft and host can lead to rapid graft incorporation and residual weakness of the construct is minimal [86].

May be attached implants and osteointegration should progress more fast. Radiographic presentations of union within twenty weeks has been reported and the he distal aspect of the canine ulna can be used as a vascularized transposition autograft to replace distal radial defects, and viability can be maintained [97, 107, 110].

Differences in graft incorporation between cortical and cancellous grafts become evident during the stage of graft revascularization and remodeling. The process takes longer for cortical bone. Revascularization takes approximately 2 months, in humans, because of the structure of the cortical graft, which does not allow as large a contact area for vascular penetration between the graft and the host [86, 111, 112].

In contrast to cancellous grafts in which incorporation is initiated by new bone formation, the osteoclasts must first initiate resorption of the dense cortices in cortical grafts to allow revascularization. In dog studies, it has been shown that cortical grafts have significantly decreased strength at 6 weeks that remains low through 24 weeks but returns to normal strength by 48 weeks after transplantation [112].

Although not being osteogenic due to the processing they undergo in order to decrease their antigenicity, allografts provide a natural bony scaffold, eliminate the need of a second surgery, may be osteoinductive (depending on their preparation), and are osteoconductive [97].

Despite these unique characteristics, autografts have been increasingly replaced by the synthetic bone substitutes because of the high morbidity rate (20%) associated with the second surgery for obtaining autograft and because the quantity of available bone is often limited [113].

Allografts

Allogenic bone (allograft) is sourced from a different patient of the same species. Allografts is often the most preferred alternative to autografts, being the most used bone substitute in Europe. They support bone formation due to its osteoconductive and osteoinductive properties and are easily incorporated into the bone structure [97].

Furthermore, allografts have several advantages over autografts, as they eliminate the need for a second surgical site, the limited amount of bone graft that can be collected from the patient and they are available in customized forms.

However, they also carry some limitations such as delayed vascular penetration, slow bone formation, higher rates of resorption, and the possibility of immunogenicity and disease transmission. Allografts are generally acquired through tissue banks and are available as fresh, fresh-frozen, freeze-dried, or demineralized freeze-dried forms. Bone banks seek, process, and store allografts for distribution [16].

Allograft can be obtained either from cadaveric donors or from patients undergoing primary hip arthroplasty (femoral heads recession) [40]. The allogenic bone with variable biologic properties is available in many preparations, such as, demineralized bone matrix, morselized and cancellous chips, cortical cancellous and cortical grafts, and osteochondral and whole-bone segments [103].

The safety of bone allografts has noticeably increased during the last decades, although their association with disease transmission is still a valid concern. These diseases can either originate from the donor or can be introduced during the succeeding steps from harvesting to implanting. Bacterial and viral agents, including *Clostridium*, and *Mycobacterium* have been introduced to the recipient of bone allografts [114]. However, and despite several aseptic techniques, allografts pose potential risks of bacterial contamination and spread of viral and prionic transmission diseases [40].

Additionally, allogenic bone from cadavers must be harvested carefully under rigid conditions and then properly stored in tissue banks to prevent possible immunologic complications or possible transmission of viral or bacterial pathogens. Sterilization of demineralized bone may alter the physiochemical properties critical for bone induction when methods such as gamma radiation are employed. It is recognized that irradiation of demineralized bone powder before implantation weakens the osteogenic response by approximately 20%. It is therefore extremely difficult to use natural bone as an implant, therefore remains a need for a synthetic bone replacement material [17].

Allografts are similar to autografts in that they are comprised of cancellous and/or cortical bone with greater quantities and sizes being typically available. Disadvantages of allografts include limited supplies of materials and the potential for transmission of disease. The disadvantages of the existing products require better devices and methods for treating defects in the animal live tissue [17].

Also the well-known disadvantages of allografts have favor the development of synthetic bone grafts for bone regeneration either in human or in veterinary medicine. For those reasons in recent decades there has been an enlargement of the need to use bone grafts [115]. So, these facts have motivated the development of bone graft substitutes so there has been great advances in the development of synthetic alternatives for the past decades [91, 116].

Xenografts

When the bone graft is obtained from one species to be implanted in other species it is called xenograft. Xenogenic or xenografts weren't considered an acceptable therapy in bone grafting indications but it has changed within the last years [72]. They are usually made of naturally derived deproteinized cancellous bone from other species. Xenografts were not considered an acceptable option for bone graft, but this has changed within the last 10 years as purified anorganic (deproteinized) bovine bone has become increasingly popular for certain grafting procedures, namely oral surgery [117].

The risk of adverse immunological reactions or transmission of virus and prionic diseases, such as bovine spongiform encephalopathy, are negligible because the organic component is extracted from the bone. The difficulties in manipulation and adaptation to the shape of the lesion may be other limitation of the use of autografts, allografts and xenografts [3].

Synthetic Bone Grafts Substitutes

Due to some disadvantages of autografts, allografts and xenografts (**Table 1**), in recent years, synthetic bone substitutes, mainly based on HA and TCP, have been developed in the treatment of bone defects [118]. Over the time the concept of applying biomaterials was developed by minimally invasive surgical techniques through small incisions with little manipulation tissue to promote a low post-operative morbidity that there may arise, as well as inflammatory complications with circulatory changes, associated with incisions and extensive manipulations [3, 119].

Table 1: Advantages and disadvantages of various types of bone grafts (Adapted from [98]).

Type of Graft / Bone Substitute	Advantages	Disadvantages
Autograft (Autogenous bone graft)	Osteogenesis Osteoconduction Osteoinduction Non-immunogenic	Increases local morbidity in the patient: pain and infection in the donor area, visceral lesion during collection; Lack of vascularization; Quantity and limited availability.
Allograft and Xenograft	Osteoconduction Osteoinduction Less local morbidity	High cost; Risk of disease transmission; High risk of rejection; Lack of osteogenesis and vascularization.
Manipulated substitute using biological or synthetic materials	Possibility of incorporating growth factors and stem cells that promote osteogenesis and incorporation of the graft; Can be molded to fill the defect, through 3D printing; There is no local morbidity in the patient; High quantity and availability.	Poor neovascularization; Limited mechanical properties; Unknown immune response; Osteogenesis is limited in the porous materials (as a result of the manufacturing process).

The architecture of the material shows the presence of pores, macropores, micropores and interconnected pores is another crucial feature of bone substitutes, because this type of structure allows the invasion of blood vessels osteoblasts, endothelial cells and cells with regenerative potential like mesenchymal stem cells (MCSs), hematopoietic progenitors cells, and others, present in some tissues including the bone marrow [120].

The main morphological requisites for allowing bone ingrowth are the existence of open and interconnected pores, with pore diameters larger than 100μm for proper vascularization. The interconnectivity of the pores can be achieved by

adding pore formers with a compromise between interconnectivity and mechanical strength [121].

This type of structure also allows the local supply of nutrients and removal of metabolic cell products, promoting bone invasion and resorption of the biomaterial [120, 122, 123].

Porous bioceramics can be produced by several techniques such as the polymeric sponge technique, foaming processes and techniques using organic additives [121]. There is a threshold at which the increase of pore size is detrimental to bone regeneration with problems in osteointegration and bone contact with biomaterial [124] and the excessive diameters may obstruct the filling the pores of cells [125]. The ideal scaffold have a balance between mechanical properties, a porous architecture, and degradability while remaining osteoconductive [126]. A reasonable biodegradation rate and osteoconductivity are desirable for bone grafting materials [127]. The size of the porosity is also important as regard to the rate of degradation of the biomaterial because higher porosities on very degradable materials, may lead to rapid absorption, resulting in a loss of function of the implant before the growth of new bone [120].

Macroporosity has an important impact on osteogenesis and microporosity is undoubtedly another fundamental factor that one has to take into account in producing synthetic bone grafts [120]. Macroporous are pores with sizes more than 150 μm and micropores less than 10μm. The presence of micropores and its increasing number causes the increase of the contact surface area. For bone regeneration using these biomaterials, the activity of precursor cells is dependent on the anchoring to the surface of the biomaterial in order to differentiate, proliferate and initiate production of mineralized bone matrix [128].

Synthetic bone graft materials available as alternatives to autogenous bone for repair, substitution or augmentation include: metals, resorbable and non-resorbable polymers, inert ceramics (e.g., alumina, zirconia), special glass ceramics described as bioactive glasses, calcium sulfates, calcium carbonates, and calcium phosphates (CP) [129].

There are a variety of techniques available to the surgeon to manage local bone loss, each with their own advantages and disadvantages. It is well known that there is morbidity associated with harvesting of autogenous bone graft and limitations in the quantity of bone available. Alternatively, allografts have been reported to have a significant incidence of postoperative infection and fracture as well as the potential risk of disease transmission. During the past 30 years a variety of synthetic bone graft substitutes has been developed with the aim to minimize these complications. The benefits of synthetic grafts include availability, sterility and reduced morbidity [130].

Bone-graft materials usually have one or more components: an osteoconductive matrix, which supports the ingrowth of new bone; osteoinductive proteins, which support mitogenesis of undifferentiated cells; and osteogenic cells (osteoblasts or osteoblast precursors), which are capable of forming bone in the proper environment [103, 131].

Synthetic bone substitutes are used in a number of clinical applications, such as post-traumatic complications of bone fractures, bone defects, arthrodesis, gaps or removal of bone tumours, advanced age, etc. A bone graft should have specific characteristics depending on its application. If a rapid bone attachment is required, a highly bioactive material should be used [132].

The goal of synthetic bone substitutes is to mimic the biological properties of the natural bone. Therefore, the morphology and properties of the natural bone should be used as a standard that must be met by the ideal bone substitute [133]. These substitutes have several advantages against autografts or xenografts, namely their unlimited availability and the fact that they eliminate the risk of disease transmission [118]. For the graft or bone substitute to be successful, there are three basic criteria, namely, osteogenesis, osteoinduction and osteoconduction [134, 135].

Osteoconductive capacity is to route the graft blood vessel formation with the consequent creation of new bone through the graft. Osteoinduction is the ability of the graft to stimulate undifferentiated mesenchymal stem cells (MSCs) neighboring the implant tissue to differentiate into osteoblasts and begin to produce bone tissue. Osteogenesis implies the existence of living osteoprogenitor cells in the graft, which can proliferate/differentiate into osteoblasts and osteocytes. Finally, osteointegration refers to the ability to interconnect to the original graft bone [72, 95, 96, 136].

Bone grafts are required to restore skeletal integrity and enhance bone healing in several orthopedic and maxillofacial surgical procedures, but its use depends on its availability in the patient (autograft) or in tissue banks (allograft or xenografts). The incidence of orthopedic disease has increased, not only due to increased life expectancy but also due to the rising popularity of sporting activity, leading to traumatic lesions [3].

As a result, the last few decades have seen an increasing need for the use in many situations of bone grafts in human medicine and has extended into veterinary medicine. Currently bone is the second most commonly transplanted tissue, exceeded only by blood [3, 62, 96, 113, 137, 138].

In recent decades, these limitations have led to a systematic and very intense demand for synthetic bone substitutes, HA [$Ca_{10}(PO_4)_6(OH)_2$], calcium sulfate (CS) [$(CaSO_4)$], tricalcium phosphate (TCP) [$(Ca_3(PO_4)_2)$] and coral-based materials. Synthetic bone graft substitutes are relatively abundant, easy to manufacture, and eliminate many of the major problems associated with autografts and allografts. The use of synthetic materials has intensified in orthopedics and maxillofacial and periodontal trauma surgeries [139]. Moreover, the transmission of infectious diseases through the allograft, either in human medicine or veterinary medicine, is a real danger [140, 141].

In companion animals, like dogs and cats, bone grafts have been used for a variety of indications, including the following: treatment of highly comminuted fractures for stimulation of bone union before implant failure, usage in animals with a poor osteogenic potential (older, debilitated or small and toy-breed patients), non-union fractures, filling of bone defects created by aneurismal bone cysts or by the performance of surgical curettage of bone following tooth extraction, and to enhance healing following ventral stabilization procedures in the cervical spine or after joint arthrodesis [96].

Bone grafts not only fill an empty space, they increase local resistance, form an extracellular matrix support and direct bone growth. Immobilization and recovery of normal anatomy are other important functions of this grafts [65]. In order to be successful in clinical applications there are important stages of bone regeneration

that a bone graft must fulfill: osteogenesis, osteoinduction, osteoconduction and osteointegration, as previously referred [96].

Bone grafting is based on the fact that bone tissue, unlike most other tissues, can regenerate completely if provided the space into which to grow. As new bone grows, it will generally replace the graft material completely, resulting in a fully integrated region of newly formed tissue [142]. After implantation, the bone reacts towards the biomaterial in a variety of ways depending on the material type. This reaction depends on the tissue response to the implant surface [143, 144].

There is a huge variability of bone injuries resulting in different biologic needs and biomechanical forces which should be evaluated by the surgeon. The selected material should meet those requirements, while minimizing costs and complications. Replacement of extensive local bone loss is a significant clinical challenge [130].

Current research regarding bone grafts includes the development of grafting techniques and the controlled release of active substances such as drugs, bone growth promoters, antibiotics and cell therapies to maximize chance of a successful graft [145, 146]. Those limitations presented in the past few decades, lead to a systematic and very intense search for biomaterials that can be used as synthetic bone substitutes, causing the clinical use of such biomaterials to annually grow [96, 139].

Since biomaterials will interact with the surrounding tissues after their implantation site, the cytocompatibility and the histocompatibility are very important issues to be tested before clinical trials are conducted, as previously referred. A side from interactions of various complications such as rejection of the material by the body, with surrounding tissue death of the implant site, material corrosion with release of toxic chemicals, fragility of the material over time with consequent loss of function. The main requirement of a biomaterial is biocompatibility, property on which the foreign material provides an adequate response when implanted in living tissue without damaging surrounding tissue [65].

An ideal bone graft should be reabsorbed, confer resistance similar to cortical bone, and have osteoconductive properties, osteoinduction be easy to manipulate, sterilizable and not cause reactions adverse. However, there is no biomaterial that can cover all these properties [96, 147, 148]. It should be always considered, that a

biomaterial implanted in a living body, triggers a series of reactions that are dependent on the type of material used [65, 72, 90, 149, 150].

There are numerous types of synthetic bone grafts, mostly divided into ceramics, polymers, metals and composites [3, 147, 151]. So, after implantation, the bone reacts towards the biomaterial in a variety of ways depending on the material type. This reaction depends on the tissue response to the implant surface. Various synthetic bone substitutes have been developed to reconstruct the bony defects that clinicians often encounter during surgical procedures. Among various synthetic bone substitutes, calcium phosphate ceramics have been investigated because their composition and structure are similar to those of human bone [152, 153].

In this work will be discuss mainly of ceramics used as synthetic bone substitutes applied in animal models and in pre-clinical and clinical cases in small animal's veterinary medicine.

Ceramic Bone Grafts

The ceramic inorganic non-metallic solids are prepared by action of heat and subsequent cooling. This group of materials includes: HA, TCP and CS, derived from coral, alumina (aluminum oxide) and zirconia [62, 154].

There are numerous ceramic systems that display both biocompatability and degradability. In the body, the bone itself is the natural storehouse of minerals. The major mineral component of bone is HA, a form of CS. Other calcium phosphate salts in bone include monotite, brushite, calcium pyrophosphate, TCP, octocalcium phosphate, and amorphous calcium phosphate [17].

The biological behavior of ceramics, besides being dependent on the chemical composition is also influenced by their physical and morphological characteristics [3].

The first material bind to the bone was a bioactive glass which is successfully used as synthetic bone substitute. The formation of a biologically active hydroxyl-carbonate apatite layer (HCA) over the exposed surface, which is chemically and structurally similar to the mineral phase of bone allowed this connection [155].

In the market various ceramic are available with different shapes and dimensions. One of the critical points in filling bone defects with a biomaterial is the adaptation to the defect. The possibility of using granular multi-particulate forms allows the biomaterial to adapt to any defect geometry, especially when associated with an appropriate vehicle. These granular forms maintain a space between the particles (inter porosity), which is invaded by a vascular network, thereby increasing the capacity of bone growth and implant resorption [156].

It is predicted a great future growth of synthetic bone substitutes mainly due to the development of composites in tissue engineering, namely composites containing osteogenic cells and growth factors [157]. Within the synthetic materials, the ceramics present as the main type of biomaterial for the bone repair due to its resemblance to the minerals of the natural bone. Ceramics are considered biocompatible materials with low tensile properties, with excellent compression strength, high load resistance and low frictional properties [158]. Synthetic bone substitutes are available in the market in a wide variety of textures, sizes, shapes (blocks, granules, cements, gels and strips) and composition (**Table 2**) [159].

Table 2: Listing of some ceramic synthetic bone substitutes available on the market (Adapted from [160]).

Product name	Features	Company
Hydroxyapatite		
Ceros80®	Dense Hydroxyapatite polycrystalline	*Straumann®*, United Kingdom
Biocoral®	Natural mineral skeletons of the coral *Scleractinian*	*Inotebm®*, France
Pro Osteon®	Coralline macroporous Hydroxyapatite: 55% porosity	*InterPore®*, EUA
Osteograf®	Dense Hydroxyapatite polycrystalline	*CeraMed®*, EUA
Calcitite®	Dense Hydroxyapatite polycrystalline	*Calcitek®*, EUA
Tricalcium Phosphates		
Chronos®/Ceros®	Tricalcium Phosphate: 60% porosity	*Mathys®SA*, Switzerland
Biosorb®	Tricalcium Phosphate: 45-70% porosity	*SBM®S.A*, France
Vitoss®	Ultraporous Tricalcium Phosphate: 88-92% porosity, interconnected pores	*Orthovita®*, EUA
Cerasorb®	Tricalcium Phosphate: 65% porosity	*Curasan®*, Germany
Bioactive Glass		
Bioglass®	Bioactive glass: 90-95% porosity	*Novabon®*, EUA
Ceravital®	Group of glasses and ceramic glasses with several compositions	*Bromer®*, Germany
Biogran®	Bioactive glass	*Orthovita®*, EUA
Hydroxyapatite or Glass Reinforced Tricalcium Phosphates		
Actifuse®/Pore-Si®	Si-HA, 80% porosity, interconnected pores	*ApaTech®, Ltd.*, United Kingdom
OsteoStim®	Si-FTC, 60-75% porosity, interconnected pores	*EBI Medical®*, EUA

The bioceramics are provided as commercial products in form of granules, blocks, putties, self-setting cements or as coatings on metal prostheses, e.g. for the treatment of bone defects [161].

The importance of optimizing the loading and distribution of ceramic particles for instance in a polymer/ ceramic composites was proved and maximized bone healing. The main advantage of this combination is the osteoconductivity of the ceramic component with the ductility of the polymeric component, resulting in a graft that meets many of the desired properties for bone void fillers [162]. Moreover, an injectable compression-resistant bone graft was developed and resulted in a comparable volume of new bone formation with the clinical control [163].

Calcium Phosphates

Due to their abundance in nature and presence in living organisms, calcium apatites and other calcium orthophosphates remain the chemical compounds of special interest in many fields of science, including geology, chemistry, biology and medicine [62, 164].

Calcium phosphates (CP) biomaterials including biphasic calcium phosphate (BCP) ceramics have proven efficacy in numerous clinical indications. Their specific physicochemical properties (HA/TCP ratio, dual porosity and subsequent interconnected architecture) control (regulate/condition) the progressive resorption and the bone substitution process [100].

These inorganic materials differ in composition and physical properties from each other and from bone. According to their composition, synthetic calcium phosphates are classified as HA, $Ca_{10}(PO_4)_6(OH)_2$; alpha- or beta-tricalcium phosphate (α- or ß-TCP), $Ca_3(PO_4)_2$; biphasic calcium phosphates (BCPs) for mixtures of HA and ß-TCP; and un-sintered apatites or calcium-deficient apatites [100].

Their chemical similarity to the inorganic component of bone and teeth, biocompatibility, bioactivity and osteoconductivity, Calcium Phosphates (CP) $Ca_{10}(PO_4)_6(OH)_2$ ceramics including the β-TCP, HA and biphasic calcium phosphate (BCP) are often considered as bone grafting materials, either alone or as autograft extenders [165]. BCP ceramics were used successfully in human surgery because their

chemical composition is closely related to human bone mineral [166]. For more than twenty years, calcium phosphates ceramics like β-TCP and BCP have been described with success as efficient and safe for sinus floor augmentation in humans [167].

CP, a major component of natural bone, has been used in medicine and dentistry for over 20 years. HA is a CP that makes up most of the mineral phase of the bone. It is biocompatible, osteoconductive with an excellent chemical and biological affinity with bony tissues. Many studies with animals have evidenced the long-term biocompatibility of solid HA and its favorable interaction with soft tissue and bone [75, 168].

CP ceramics such as HA, TCP, dicalcium phosphate (DCP) ($Ca_2P_2O_7$) and tetracalcium phosphate (TeCP) ($Ca_4P_2O_9$), and others) are graft materials used in all over the world in the bone tissue regenerative process [134, 169-171]. The main reason for this success is that this type of synthetic material mimics the mineral phase of bone due the similarity of these compounds to the natural bone tissue apatite [136].

Actually, HA and TCP are the most widely studied of the CP, which have calcium to phosphate ratios of between 1.5 and 1.67 respectively. Those particular CP ceramic is known as a physiologically acceptable biomaterial which is useful as a hard tissue prosthetic [17]. Most commercial products are made from either HA or ß-tricalcium phosphate [161].

Recent studies explain that the main advantage of CP prove no interference on the part of the calcium and phosphate ions with cell function and physiology of the surrounding tissue, mimicking the mineral phase of bone, due to the similarity these compounds with apatite in the natural bone tissue. Moreover during the dissolution of the material, release of such ions can act as a catalyst for bone formation and precipitation of apatite layer adjacent to the surface of the material [3, 62].

Despite CP being osteoconductive, the conductive bone is restricted to areas nearby the host bone, limiting their clinical application in the repair of critical-sized bone defects. However, a subgroup of CP ceramics with special physicochemical properties was found to trigger bone formation in heterotopic sites (e.g. intramuscular or subcutaneous) i.e. osteoinduction [165].

Synthetic Hidroxyapatyte

Synthetic HA, $Ca_{10}(PO_4)_6(OH)_2$, is one of the CS used more frequency as bone substitute. It is a compound with a great bioenerte osteointegration, but fragile and low resorption rates (5-15% per year) [96, 172]. HA has a similar structure to the bone mineral phase, with osteoconductive properties, biocompatibility and bioactivity by binding directly to bone without the mediation of fibrotic tissue [173-176].

The presence of capillary blood vessels inside the macropores of HA ceramics is associated with the process of osteoinduction. These blood vessels could be a source of progenitor cells, which can differentiate into osteoblasts under the stimulation of the inflammatory cytokines released by macrophages [177].

HA does not provide mechanical properties that confer resistance in together with long stay in the body without a refurbishment and replacement, undertake the restoration of the normal resistance bone, causing bone to remain for long periods and fragile subject to fractures [96]. These limitations led to the development of formulas to improve resorption of HA. These changes consist of modifying the HA, combining it with substances that increase the rate of absorption [175].

Using of HA as a dental implant or graft supplement has proven to be a good material for alveolar ridge augmentation, pulp capping, and filling of periodontal defects, where the use of autogenous graft has been found to be less than optimal [178].

In Human Medicine due to the significant increase in dental implants placements and in alveolar ridge augmentation procedures over the last two decades there was a development of a great demand for bioactive bone substitutes with higher resorbability, which enhance osteogenesis at the same time [153].

Also bone fractures at weight-bearing sites are challenging to treat because the difficulty in maintaining articular congruency. So an ideal biomaterial for fracture repair near articulating joint sets rapidly after implantation, will stabilize the fracture associated with a minimal rigid implants, stimulates new bone formation and remodels at a rate that maintains osseous integrity [179].

Bioceramics composed of 100% HA provide high strength to loading but are not resorbed and reside in the defect for several years after callus formation. On the other hand, scaffolds mainly composed of tri-calcium phosphate have a greater capacity to be resorbed but they are too fragile to sustain the weight load [126].

In human patients several advantages are associated to HA as a dental implant such as, availability, biocompatibility, absence of antigenic response, ability to combine with anesthesia, low infection risks or permanent hyperesthesia, lack of significant resorption, high rate of good results, and no need for perfect oral hygiene on the part of the patient [178].

Tricalcium Phosphates

Other CS which is widely used in clinical applications for bone regeneration is TCP, ($Ca_3[PO_4]_2$), containing 39% calcium and 20% phosphorus composition. This composition is similar to the mineral part of bone The TCP is more soluble than HA with a higher rate of *in vivo* absorption [90, 95, 180]. The biomaterial rate of resorption should be at the same time of the formation of new bone. Slow rates of absorption cause the biomaterial to remain in the same place and preventing the growth of new bone as with the HA. The TCP absorption rate is faster than the rate of new bone formation, leading to void spaces, weakening the location of implant [130].

Interconnections between the implant and new bone are essential to strengthen and stabilize the site of injury. Ideally the new bone should connect to the implant through direct connections with the formation of carbonated apatites in a single interface. The high absorption rates of TCP may compromise the training these direct links [90]. TCP has two crystalline phases: alpha (α-TCP) and beta (β-TCP). The β phase is more stable and has a slow rate of absorption. The high rate of solubility of α-TCP will allow for bone grafts release active substances such as drugs or growth factors that promote osteogenesis [90]. TCP ceramics are used as bone void fillers because of their bioactivity and resorbability, while their performance in bone regeneration and material resorption vary with their physical properties, e.g. the dimension of the crystal grain [181].

Biphasic Calcium Phosphates (BCP)

An ideal biomaterial for bone regeneration must connect directly to the bone, and be reabsorved at a rate equal to the rate of new bone, so as implant integration and gradually replacing it with new bone, between the final replacements of new bone by the implant structure be weakened. So to overcome the disadvantages of poor absorption of HA and rapid absorption of TCP, developed the concept of biphasic material, resulting from the combination of these two substances [168, 182].

This type of biphasic material allows adapting the rate of absorption with bone growth, altering the ratio of HA / CP. Different ratios HA / CP have been proposed, with the rate of resorption inversely proportional to this ratio [72]. Another advantage of this type of biphasic compounds comes from the possibility of faster absorption TCP lead to precipitation ion calcium phosphate, which induces the formation of direct links between the biphasic compound and new bone. To improve the bioactivity and bone regeneration, these biphasic compounds may be supplemented with trace elements such as magnesium, fluoride, sodium zinc and silicon thereby obtaining a more similar chemical composition to the mineral phase bone [135].

BCP ceramics are now successfully used for bone substitution in many different clinical situations such as repair of bone defects, bone augmentation in spinal arthrodesis, periodontal treatment, or as coatings for metallic implants [100].

Chapter 2 – Aims

The doctoral thesis was designed in order to develop effective methods to enhance the bone regeneration capacity of Bonelike® spherical granules between 250 and 500μm when combined with cell-based therapies of human mesenchymal stem cells isolated from the adult human dental pulp (hDPSCs).

In order to achieve these goals, the following specific objectives of this doctoral project were designated:

1. Develop the cell-based therapies from human adult dental pulp (hDPSCs) (xenografts) to associate with the bone substitute, including its *in vitro* characterization (cytogenetic analysis for evaluation of karyotype, flow cytometry, immunocytochemistry, and tri-lineage differentiation capacity and osteogenic gene expression by RT-PCR) for their application *in vivo* as pre-clinical trials.
2. Testing *in vivo* (sheep model) the patented bone substitute Bonelike® (JD Santos, GW Hastings, JCKnowles, European Patent WO 0068164) in the form of spherical microporous granules between 250 and 500μm associated with hDPSCs.
3. Testing *in vivo* (in selected clinical cases of orthopedic surgeries in small animals) the patented bone substitute Bonelike® (JD Santos, GW Hastings, JCKnowles, European Patent WO 0068164) in the form of spherical microporous granules between 250 and 500μm.

This project intended to contribute to the development of new tissue engineering strategies, in the context of bone regeneration, targeting a rapid restoration of the skeletal integrity and reduction of the associated morbidity where biomaterials and cellular therapies play an important role. Also, in this project, a multidisciplinary team and longitudinal research approach were the key for the development of the biomaterial and cell-based therapy, the *in vitro* characterization of the developed cellular system, the pre-clinical testing in an appropriate animal model (ovine) and, finally, a clinical trial in small animals. The final goal was obtaining an adequate bone substitute, which effect would be improved by its association to MSCs from the dental pulp (hDPSCs) to be used in Veterinary and Human Medicine for bone regeneration.

Chapter 3 – Bonelike®

Introduction

Large bone defect repair is a difficult problem to be solved urgently in the orthopedic field and the application of bone repair materials appears as a viable alternative. Recent development of calcium phosphate ceramics and other related biomaterials for bone graft provide improved control of the process of resorption and the ability for new bone to form. Glass-based ceramics, such as bioactive glasses, and calcium phosphates are popular materials for mineralized tissue regeneration [73, 183].

Bonelike ®

Santos et al. [184-186] developed a method of producing a synthetic bone substitute composed of two types of materials: HA and P_2O_5-CaO After being subjected to heat treatment, they give rise to a modified HA with a secondary matrix β and α-TCP. Its main functions are bone adhesion, cell proliferation and differentiation. Bonelike® is reabsorbed slowly and controlled and contributes to the natural remodeling of the bone. This final product has already been patented and registered as Bonelike® exclusive property of Biosckin® Molecular and Cell Therapies S.A., and it is available in the form of blocks, dense granules and interconnecting macroporous 3D structures, similar to human bone, in order to allow bone growth (**Figure 8**) [118, 187-189].



Figure 8 – Bonelike® in the form of blocks, dense granules and macroporous 3D structures (Adapted from [187]).

This biomaterial may be regarded as a phosphate biphasic or triphasic in case β-TCP and α-TCP are considered separately. The α- and β- phases of TCP are dispersed homogeneously in HA matrix due to the presence of a liquid phase during the sintering, resulting in improvement of a microstructure superior mechanical properties and bioactivity relative to pure HA. The incorporation of glass incites the decomposition of the HA part to a secondary phase, β-TCP. Successively, at higher firing temperatures this phase change to α-TCP [186, 190, 191].

Through human endothelial cells viability tests, it was possible to conclude that Bonelike® allows the adhesion and expansion of these cells, and also the subsequent proliferation with the phenotypic expression of endothelial cells [136]. Subsequently numerous pre-clinical trials were performed using animal models such as the rabbit and sheep [122, 133, 173, 192]. They began by comparing the behavior of Bonelike® coated implants and HA-coated implants in the rabbit tibia. With this study, it was possible to conclude that the Bonelike® coated implant was more osteoconductive, since the bone formed faster when compared to HA [65].

Still in rabbits, circular defects were generated in the femur and filled with Bonelike® polygonal granules of 150 to 250 μm and 250 to 500 μm. Through radiological examinations, histological studies and analyzes by scanning electron microscopy (SEM) it was found that after 12 weeks the Bonelike® surface was almost completely surrounded by new bone. It was concluded that this synthetic bone substitute was highly osteoconductive and with good potential to be used in clinical cases of maxillofacial reconstructive surgery and orthopedics [159].

Through the combination of Bonelike® with Floseal® and raloxifene hydrochloride, it was also possible to conclude that osteoconductive properties of the material are not significantly affected by the presence of the vehicle and the therapeutic molecule. The association of Bonelike® to a resorbable vehicle (such as Floseal®) can act as a system of controlled release of osteoinductive molecules, thus increasing osteointegration, as well as the possibility of incorporating several therapeutic molecules, such as bone morphogenetic proteins (BMPs), antibiotics and anti-inflammatories [192].

After the application of Bonelike® in rabbits was verified, the improvement of the pre-clinical studies to a more adequate and closer to human anatomy and

biomechanical forces animal model was necessary. For that reason, these pre-clinical trials were developed in the ovine model, since the ovine present a body weight more similar to humans. Bonelike® in the form of polygonal granules (from 150 to 250 μm) was applied with a CS carrier in the ovine femur. This study confirmed the osteoconductive properties of Bonelike® and found that the combination of Bonelike® with calcium sulfate allows the fixation and stabilization of this bone substitute when applied in bulky defects [156].

Bonelike® was also used in human clinical trials where Bonelike® cylinders were implanted on the lateral side of the tibia when the osteotomy was performed in the surgical treatment of compartmental knee osteoarthritis [102]. With this study, it was concluded that this material was suitable for use in humans and had osteoconductive properties and adequate osteointegration [40, 102, 151].

This bone substitute was also used in several clinical applications such as, in dental surgery, namely in implantology after surgical removal of included teeth, filling of bone cysts extraction of two or more walls, as well as bifurcations or trifurcations of teeth, bone elevation of the maxillary sinus and coating of titanium dental implants [64, 134, 193]. In some clinical cases, the extraction of a large segment of the jaw was necessary to ensure complete removal of the lesion, leading to massive bone loss [130].

The main indications for Bonelike® orthopedics applications include non-unions, delayed unions, mal unions, bone defects, bone cysts, tumors and arthrodesis [194] and its use has been reported in craniofacial, oral/maxillofacial [195]. The clinical applications in maxillary bone defects indicated good bonding between newly formed bone and the Bonelike® granules [196]. Currently, Bonelike® is applied in humans, in clinical cases of bone loss caused by trauma, aging or bone diseases [40, 102, 130, 192, 197].

Composition

Bonelike® is a modified calcium phosphate containing controlled proportions of HA, TCP and ionic species, which mimics the chemical and structural composition of human bone (**Figure 9**) [130].

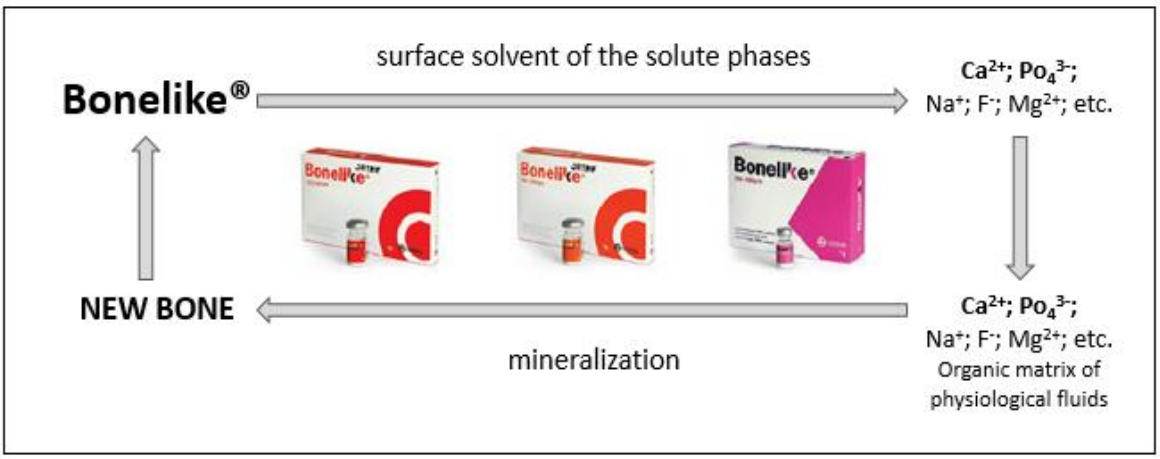


Figure 9 – Bonelike® Bone Repair Mechanism.

Pure-phase HA allows long-term stability, thus leading to increased cell proliferation and phenotypic expression. HA has a long-term resorption due to its stable structure. These ceramics have a low reabsorption rate, which implies a less formation of vital spaces for growth and bone replacement during the regenerative process. Pure Phase Beta-Phosphate (β-TCP) is more soluble than pure-phase HA. [198].

The β-TCP composites is an adequate bone substitute reporting biocompatibility, biodegradability and osteoconductivity [199]. However, rapid dissolution of β-TCP does not accompany the natural process of bone remodelling. Cellular proliferation does not accompany the rapid reabsorption of β-TCP, thus not occurring the adhesion of osteoblasts necessary for bone regeneration. The β-TCP ceramics decrease the adhesion and expression capacity of osteoblasts [198].

Bonelike® is thus a three-phase HA, α-TCP and β-TCP material that encompasses the physicochemical properties of each compound. β-TCP in terms of solubility, present intermediate solubility when compared with HA and α-TCP. Bonelike® guarantees a homogenous distribution of the TCP phases in the HA mesh, being a bone substitute whose reabsorption and replacement by new bone tissue can be controlled, depending on the percentages of HA, α-TCP and β-TCP [198].

Structures and properties

Bonelike® can be considered as a biphasic phosphate or triphasic case, when it is considered the β-TCP and α-TCP calcium. The α- and β- phases of TCP are

dispersed homogeneously on HA matrix due to the presence of a liquid phase during the sintering, which results in improvement of a microstructure with mechanical properties and bioactivity superior comparatively to pure HA [186].

The β and α phases of TCP are homogeneously dispersed in the HA matrix, resulting in a microstructure with improved bioactivity and mechanical properties relative to pure HA. Although the process begins with two different raw materials, after the heat treatment, the glass phase disappears, the final product being an altered HA matrix with secondary phases β and α-TCP [40, 114, 124, 192].

Another advantage of this methodology is that the HA / TCP ratios can be adapted by changing the sintering temperature, the amount and composition of the glass base and the presence of ions during the preparation as fluorine, magnesium or zinc [65]. By altering the proportions, it is possible to control the mechanical resistance and rate of resorption of the final product, since TCP is mechanically more fragile than HA and has a higher rate of resorption [103].

Bonelike®, when compared to HA, presents, after four weeks of implantation, an osteointegration and a mechanical resistance to flexion twice as high [65] [198]. An advantage of this methodology is that the proportions of HA and TCP can be adjusted by changing the sintering temperature, the amount, base glass composition and the presence of ions during the preparation as fluorine, magnesium or sodium. Thus it is possible to control the strength and the resorption rate of the final product, knowing that the TCP is mechanically weaker than HA and has a rate of reabsorption higher [180].

The inclusion of a base glass during the production process allows the incorporation of different ions in the HA matrix, fluorine, magnesium and sodium, approaching thus the final composition of Bonelike®, the composition of the mineral phase of bone [187, 200].

Due to its chemical composition, micropore structure and intergranular spaces among granules, Bonelike® has the potential to accelerate bone regeneration. This ceramic can also be used in several surgical procedures which demand a minimally invasive approach, due to its intrinsic injectability. These granular forms create a space between the particles (interporosity), which is invaded by a vascular network,

enhancing the bone growth and implant reabsorption. Polygonal forms can agglomerate and compact, decreasing the space between particles (interporosity), while spherical shapes prevent particles compaction, increasing the interporosity [144].

Porosity

Since the bone is a porous tissue, there is a physical reason for using porous materials to replace it. In addition, porous bone substitutes are advantageous for the early incorporation of the substitute or adherence to the surrounding bone tissue [40].

Several aspects should be considered when designing porous materials for bone substitutes: the size of the pores on the surface, the size of the mesh that connects the pores of the surface to allow the areas of bone growth to be found within the porous graft, the porosity extension, the morphology and the formation of blood vessels and canaliculi within the porosities [201, 202].

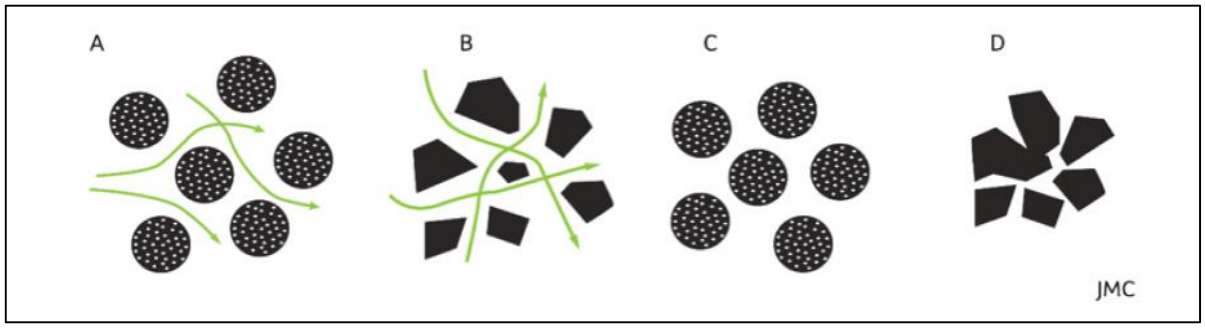


Figure 10 – Drawing to show the interporosity (space between particles) of granular forms (green arrows) A and B. Polygonal forms can agglomerate and compact, decreasing the space between particles (interporosity), while spherical shapes prevent particles compaction, increasing the interporosity, C granular form and D polygonal form (Adapted from [82]).

The portion should be open and interconnected, greater than 100 μm (minimum size for cell migration), neovascularization, water circulation, nutrients, gases and removal of metabolic wastes (**Figure 10**) [203].

The microporosity of Bonelike® increases cell adhesion (cellular anchoring) and macroporosity allows for bone growth accompanied by vascularization within the bone substitute. Both are decisive factors in increasing the rate of formation of new bone at the site of implantation. Micro and macroporosity and the degree of interconnectivity of Bonelike® pores directly affect the diffusion of gases and nutrients

present in physiological fluids, as well as the removal of residues [187]. The bone substitute acts as a structural bridge for bone regeneration as the cells grow within the porous channels [187].

Preparation and characterization of Bonelike® (spheres with granulometry between 250-500 µm)

The production of Bonelike® was preceded by the manufacture of the raw materials carried out in the materials laboratory of the company Biosckin, Molecular and Cell Therapies, S.A.®. Subsequently, the procedures for the production of HA and bioglass are described. [187].

Briefly: HA and P_2O_5-CaO based glass were individually prepared and mixed. HA was prepared through a precipitation method consisting of the reaction between calcium hydroxide ($Ca(OH)_2$) and ortho-phosphoric acid ($H_3(PO_4)_2$). A P_2O_5-CaO based glass with the composition of $65P_2O_5$- 5CaO-$10CaF_2$-$10Na_2O$ in mol% was prepared from reagent grade chemicals by using a platinum crucible heated at 1450°C. Bonelike® was obtained by adding 2.5 wt % of glass HA and mixed with a pore forming agent. The mixture was extruded and spheronized and the granules sintered. Standard sieving techniques were used to obtain the 250-500 µm particle size ranges [193]. To characterize the Bonelike®, a scanning electron microscopy (SEM) was used [198].

Techniques of materials characterization

To proceed with the physical-chemical and structural characterization of the biomaterials, it is important to perform some tests. Some of the techniques to be performed are, for example, SEM morphology analysis and chemical composition analysis by energy dispersion spectroscopy (EDS).

Analysis of SEM morphology

SEM is a type of microscopy that produces images of a sample as it passes through a beam of electrons. The electrons interact with atoms in the sample,

producing several signals that contain information about the topography and composition of the sample surface [204].

The operating principle is based on the incidence of an electron beam at a point on the surface of the target sample and the subsequent collection of the electronic signals emitted by the target material. The samples are run sequentially by an electron beam accelerated by a voltage ranging from 0 to 40 KV, finely focused through a system of electromagnetic lenses. The interaction of the electron beam with the sample results in the emission of several types of radiation and electrons, among which are the secondary electrons used in the sample image formulation [205].

SEM was used to visualize prepared Bonelike® granules, and detail on its morphology and topography (**Figure 11**).

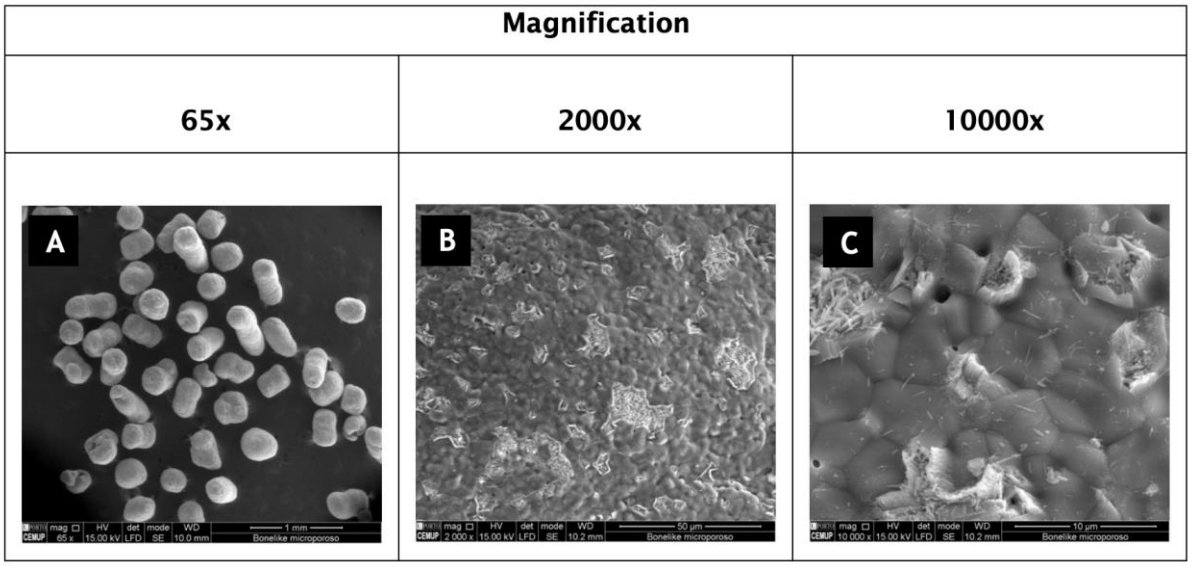


Figure 11 – SEM images of Bonelike® (Total magnification: A – 65x, B – 2000x and C – 10000x).

In order to better analyze material the mean pore diameters were measured with the aid of SEM software (**Figure 12**).

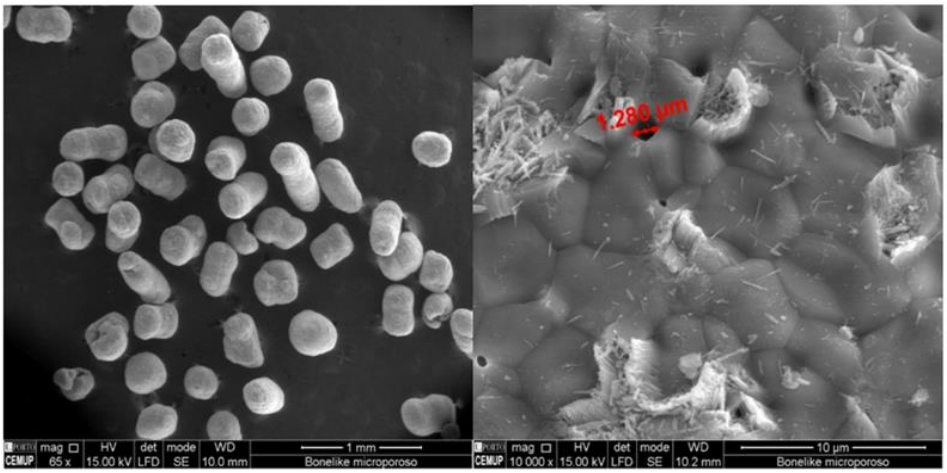


Figure 12 – Bonelike® pore size (Total magnification: left: 65x, right: 10000x).

Chemical composition analysis by EDS

X-ray energy dispersive spectroscopy (EDS) is an analytical technique used for the elemental analysis or chemical characterization of a sample [204, 206]. It is one of the variants of X-ray fluorescence spectroscopy that is based on the characterization of a sample through interactions between particles or electromagnetic radiation and matter, analysing the X-rays emitted by matter in response to the incidence of charged particles. This technique is based on the fundamental principle in that each element has a unique atomic structure, so that the emitted X-rays are characteristic of this same structure, which identifies the element [206].

Through the EDS analysis of Bonelike® (**Figure 13**), it is possible to verify that they have the same composition. Well defined peaks of oxygen (O), phosphorus (P) and calcium (Ca) are present. It is also possible to identify smaller peaks of carbon (C), gold (Au) and palladium (Pd). The presence of oxygen, phosphorus and calcium are in line with what might be expected, since Bonelike® is a modified calcium phosphate. The presence of carbon, gold and palladium is due to the coating used to make the sample conductive.

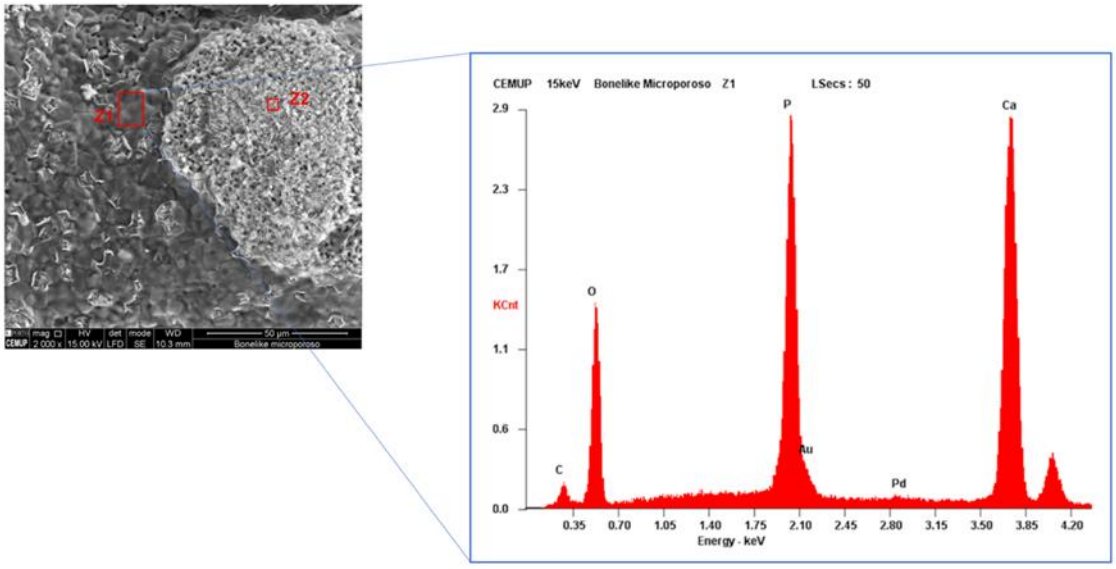


Figure 13 – Bonelike® EDS (Full image enlargement SEM: 2000x).

Chapter 4 – Human Dental Pulp Stem Cells (hDPSCs)

Introduction

In last three decades the development of cell-based therapies and their clinical application using bio-absorbable polymer matrices marked the beginning of the tissue engineering (TE). TE is an interdisciplinary field that applies principles of engineering and life sciences toward the development of biological substitutes that restore, maintain or even improve tissue function [207, 208, 209]. This is a scientific field mainly focused on the development of tissue and organ substitutes by controlling biological, biophysical and/or biomechanical parameters in the laboratory. It involves scientific areas such as cell biology, material science, chemistry, molecular biology, engineering and medicine, that seeks to repair, replace, or regenerate specific tissues or organs by translating fundamental knowledge in physics, chemistry, and biology into practical and effective materials, devices, systems, and clinical strategies [210]. Nowadays there are three well-known main pillars of TE: scaffolds, cells and regeneration factors. All of them regarded essential for the benefits of stem cells based therapies. TE is an interdisciplinary discipline addressed to create functional three-dimensional (3D) tissues combining scaffolds, cells and/or bioactive molecules. The biomaterial scaffolds, that provide structure and a protective environment for tissue regeneration; the cells that motor the regeneration itself; and the regeneration factors, that promote and enhance cell growth and differentiation [208, 210, 211].

Stem cells are immature tissue precursor cells which are able to self-renew and differentiate into multiple cell lineages. The characteristics that makes stem cells attractive as tissue regeneration motors are their ability of undifferentiated cells response with varying degrees of self-proliferation and their capacity of differentiation plasticity [212, 213].

Before birth, stem cells concentration is higher in several tissues, on the other hand, in adult tissues, there are still several “niches” with a significant number of stem cells. The adult tissues where these “niches” are already identified, include skin, adipose tissue, peripheral blood, bone marrow, pancreas, intestine, brain, hair follicles, and others, as well as in dental pulp. This last source has the advantage of

being collected from both infants and adults, which are frequently subjected to dental orthodontic treatments that imply the removal of healthy deciduous and definite teeth, respectively [213, 214].

During this thesis, our research group has been studying the isolation, expansion and *in vitro* characterization of mesenchymal stem cells (MSCs) isolated from human dental pulp (DPSCs), mainly for *in vivo* application in ovine experimental model and in clinical cases concerning companion animals. The osteogenic potential of DPSCs associated to a modified HA bone substitute was further evaluated *in vivo* in a proposed monocortical bone ovine model. The research was mainly focused on DPSCs isolated from definitive teeth, using an enzymatic method [215]. Healthy definitive teeth are a good alternative to other sources' stem cells, due to their easy collection from healthy donors and high proliferation and differentiation ability [216-219].

Mesenchymal stem cells

MSCs are thought to have promising potential in therapeutics and by definition have extensive self-renewal capacity and ability to provide additional undifferentiated stem cells and to differentiate into one or more committed descendants, including fully functional mature cells. MSCs are stromal cells that have the ability to self-renew and also exhibit multi-lineage differentiation into both mesenchymal and non-mesenchymal lineages. They are classified according to their origin, stem cells may be embryonic, germinal or somatic (foetal or adult). Moreover regarding to their capacity to differentiate into other cell types they are classified as totipotent, pluripotent and multipotent [220].

MSCs are multipotent stem cells, firstly identified in the bone marrow. They have been also isolated from multiple adult tissue sources such as cord blood, adipose and dermal tissues, synovial fluid, deciduous and definitive teeth, and amniotic fluid for several clinical applications and clinical trials, in humans and animals [221]. The considerable therapeutic potential of human multipotent MSCs has generated markedly increasing interest in a wide variety of biomedical research and clinical areas [209]. Moreover, their ability to modulate immune responses makes them an even more attractive candidate for regenerative medicine as allogeneic transplant of these cells is feasible without a substantial risk of immune rejection. MSCs secrete various

immunomodulatory molecules which provide a regenerative microenvironment for a variety of injured tissues or organ to limit the damage and to increase self-regulated tissue regeneration. Various cell type has been used for cell therapies and regenerative medicine and numerous studies have indicated DPSCs potency to differentiate into a variety of cell types such as osteoblasts, chondrocytes, myocytes, adypocytes, pancreatic β-islets cells and neuronal cells [222] (**Figure 14**).

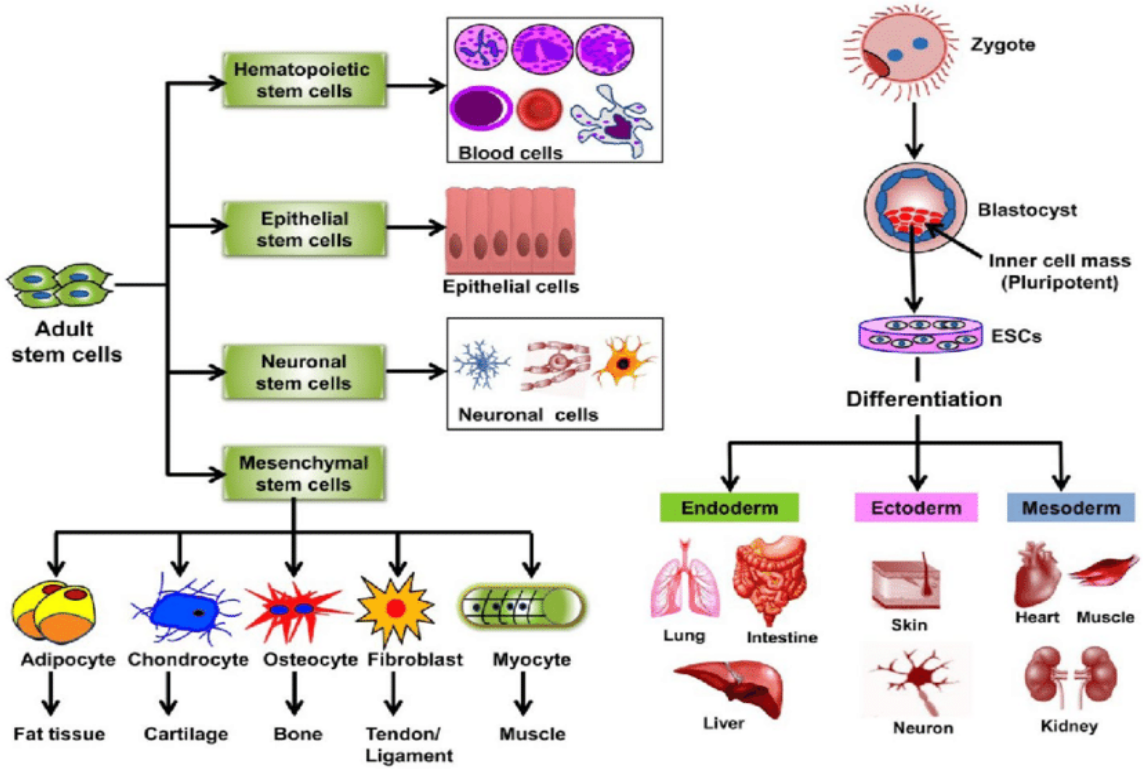


Figure 14 – Schematic diagram summarizing types and the differentiation potential of embryonic stem cells (ESCs) and adult stem cells. Stem cells potently differentiate into three embryonic germ layers, namely ectoderm, mesoderm, and endoderm [223].

Immunophenotype of MSC

Regarding to the immunophenotype of MSCs, their identification with the use of specific markers remains an important issue. There is no single surface marker, but rather a panel of surface markers which define MSCs, derived from fresh tissues or cryopreserved samples. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposes minimal criteria to define

human MSCs. First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Other than surface markers, MSCs must have ability to adhere to plastic and differentiate into osteoblasts, adipocytes, and chondroblasts under *in vitro* defined conditions. This differentiation is regulated by genetic events, involving transcription factors. Differentiation to a particular phenotype pathway can be controlled by some regulatory genes which can induce progenitor cells' differentiation to a specific lineage. Besides growth factors and induction chemicals, a microenvironment built with biomaterial scaffolds can also provide MSCs with appropriate proliferation and differentiation conditions [209].

The association of osteoconductive biomaterials with previously cultured MSCs allows the availability of bone grafts that simultaneously possess the osteogenic, osteoinductive and osteoconductive capacity, characteristics that together and in synergism of action, bring them closer to the tissues and organs that are intended to regenerate or even replace [218]. Thus, as previously referred, human DPSCs (hDPSCs) are a good alternative to mesenchymal stem cells from other sources because of their easy collection in healthy donors and high capacity for proliferation and differentiation [218, 224].

Human DPSCs in regenerative medicine

The MSCs of dental tissues instead of progenitors exhibit the minimal characterization criteria for human stem cells well-defined by the ISCT. The obtained cells, can be cryopreserved for further clinical application. The isolated and *in vitro* expanded DPSCs, can be cryopreserved for further clinical application. Finally, the obtained cell therapies, in most clinical trials, are implanted in patients, not differentiated, in order to modulate the regeneration process by local production of growth factors, cytokines, among other bioactive molecules. The application of undifferentiated MSCs has the advantage of being non-immunogenic and safe when applied in allogenic and xenogenic treatments. Disadvantages of these cell therapies, include the expensive reagents and laboratory facilities, since these cell-based therapies must be prepared in Good Manufacturing Practice (GMP) laboratories with high clinical grade reagents according to European Medicines Agency (EMA) requirements [225]. Recently, MSCs and DPSCs cell-based therapies associated to

bone substitutes are used as a promising bone graft alternatives [96]. As a matter of fact, a biological product that combines cell-based products for osteogenesis with growth factors for osteoinduction, which is associated to a perfect scaffold to maximize osteoconducion and osteopromotion is a potential treatment for bone regeneration, mainly, critical defects [225].

Dental Pulp tissue is extracted from the teeth recovered during routine dental procedure throughout the life and these teeth are the most convenient and valuable source of DPSC. Human DPSCs can be isolated from different anatomical regions of healthy teeth of young and adult individuals (deciduous and definitive dentition) and may be considered interesting alternatives to bone marrow, adipose tissue and umbilical cord matrix as sources of MSCs, which are equally or more easily isolated, *in vitro* expanded and cryopreserved for future autologous, allogenic or xenogenic clinical application (**Figure 15 and 16**) [225].

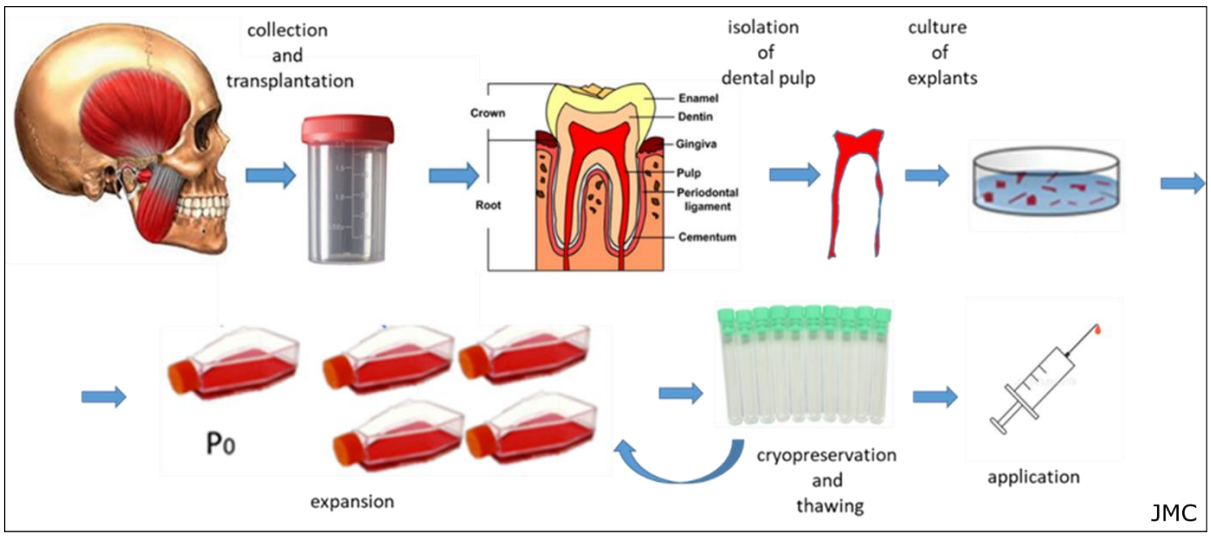


Figure 15 – Procedure performed for the isolation and expansion of hDPSCs.

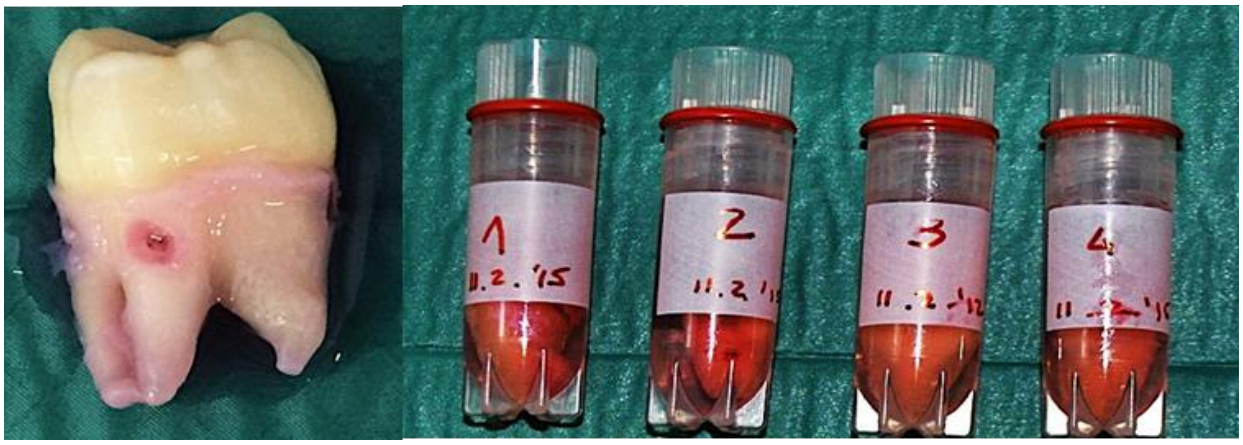


Figure 16 – Human tooth for posterior isolation of dental pulp and cryopreservation of hDPSCs [225].

Studies have demonstrated that hDPSCs have shown a potentiality for the treatment of various human diseases including dental related problems. Dental pulp is a mesenchymal-originated soft tissue that occupies the pulp chamber and root canal space and contains specialized cells on its outer surface called 'odontoblasts. hDPSC grow on highly porous natural HA scaffolds are able to migrate, adhere within the structure, differentiate and produce bone matrix. This produced matrix is completely integrated in the scaffold. As previously referred, therapies based on DPSCs can be associated with several natural and synthetic biomaterials, thus promoting bone regeneration, including non-critical and critical defects. In this case, the association of Bonelike® with hDPSCs would possibly allow better bone regeneration. In **Figure 17**, microscopic images of hDPSCs are shown during *in vitro* expansion until passage 3 (P3) – passage 4 (P4), and karyotype analysis performed previously to clinical application, in order to ensure that these cells have no neoplastic characteristics [225].

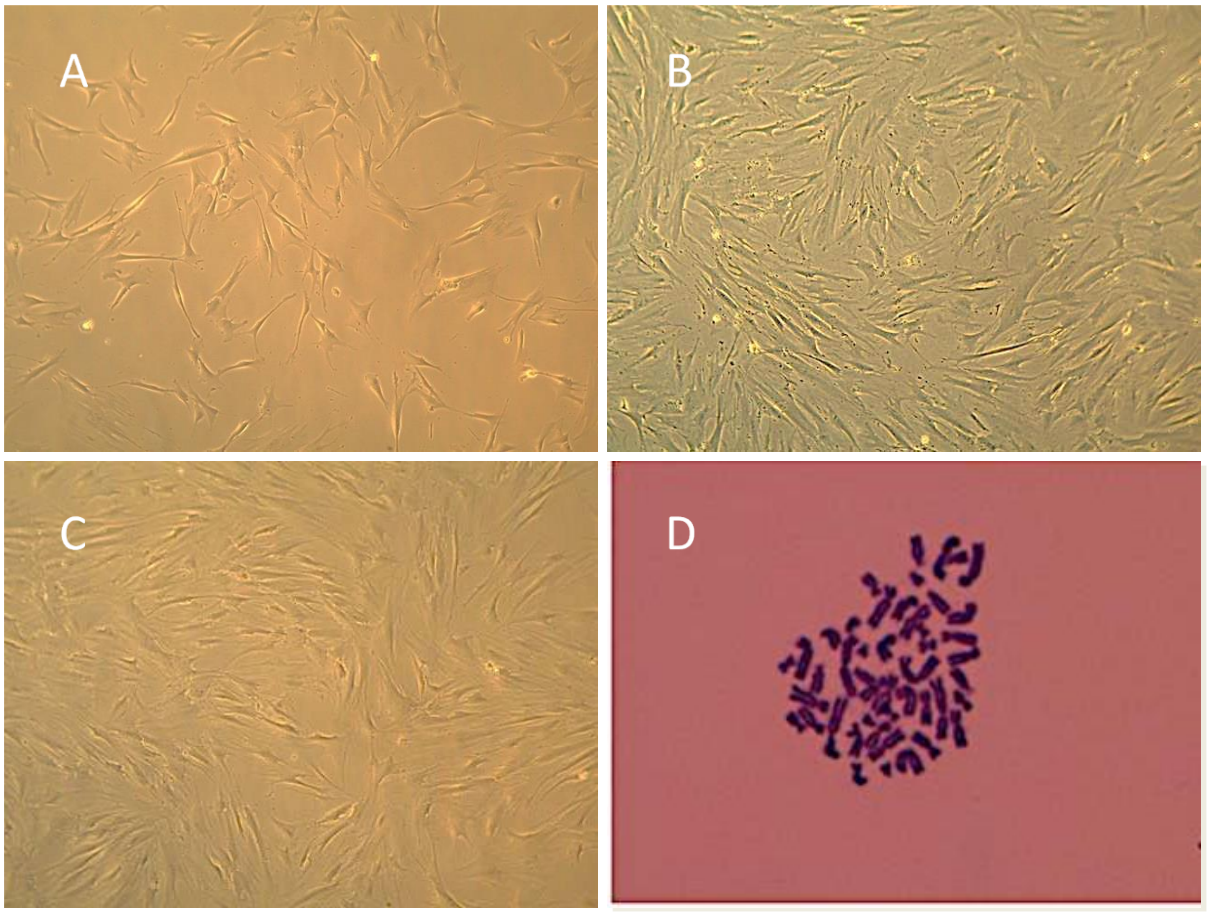


Figure 17 – (A) hDPSCs isolated from a tooth collected from a healthy adult. (B) hDPSC 48 hours after thawing and culture in alpha-MEM, glutamine and human serum. (C) hDPSC 48 hours after conditioning. (D) hDPSCs presented a fusiform morphology, similar to cultured fibroblasts (microscopic images kindly provided by the Veterinary Laboratory of Cellular Therapies from ICBAS).

In conclusion, recent advances have introduced stem cells, and specially, MSCs, as highly promising tools to provide new hope for regenerative therapies in the treatment of bone pathologies and several investigators have described various methods for isolation of DPSCs from human dental pulp. Then a successful and efficient cryopreservation of these cells can be applied for a clinical application in the near future [226].

Dental Stem Cells

DPSCs niche

An important issue in stem cell biology is the 'stem cell niche'. Human teeth are a specialized organ composed of the crown (visible part that protrudes from the gingiva), the neck (the transition area), and the root (anchored to the alveolar bone). The external layer of the tooth is composed by enamel that covers and provides

protection to the intermediate dentine part, and the inner pulp. Throughout life, human exhibit two series of teeth: the initial deciduous teeth (that start sequentially erupting around six months of age), followed by permanent / term or definitive teeth. The latest set of dentition starts developing from the tooth germ at approximately six years of age and sequentially 'pushes out' the deciduous dentition, in approximately six years (with exception for the 3rd molars or 'wisdom teeth' that, when present, arise around the twentieth year of age) [227].

There is a close relationship between the cells and the tissue local environment forms the so called 'stem cell niche' [11]. The dental tissue is an important tissue source to obtain MSCs to be used in multiple and potential Human and Veterinary clinical applications, since these cells have been isolated in several different mammal species. The dental pulp from human teeth is composed of two regions. A central vascular-nervous trunk and an external odontogenic region. The external region has four layers with distinct features and functions. The inner layer is the pulpal core, with an extensive vascular supply, that interfaces with a similar cell rich zone, where fibroblastic and MSCs populations reside. The outer layers are the cell free zone, zone of Weil, supporting capillary and nervous structures, and finally the odontoblastic layer, that interfaces with the dentin. 'Stem cell niche' is a particular structure that regulates the environment that harbours the valuable population of stem/ progenitor cells [228, 229]. In younger individuals, the dental follicle, has stem cells with higher proliferative capacity which tend to display different differentiation capabilities. On other hand, in adults, cells isolated from permanent teeth tissues and structures, have lower proliferation capacity. The quantity of stem cells in their niche and their regeneration potential is highly dependent on the age of the organism. In order to maintain tissue homeostasis, cell population is recruited in a response to a stimulus, for instance an injury or disease, to regenerate the damaged tissue and assure the host organ functions and interactions of tissues with the surrounding environment: neighbouring cells, extracellular matrix, and soluble signalling molecule, among other factors. This allows to modulate cell proliferation, survival, migration, fate, and aging [230].

The dental pulp has an age progresses, it means for example that young rats' dental pulp contains higher cell/blood vessels ration than older ones, the DPSCs present increased proliferation and colony forming units (CFU) capacity, but decreased mineralization potential [231]. For the human species, some authors report

no significant differences in expansion and bone differentiation potential of young (under 30 years old) and aged individuals (from 30 to 45 years old). The addition of conditioned medium from *in vitro* expansion of young DPSCs, enhanced adult DPSCs proliferation and diminished its osteo-differentiation capacity, while adult cells conditioned medium had the opposite effects on adult DPSCs, reinforcing the idea of niche modulation on stem cells activities, properties and potential and of the importance of the growth factors and bioactive molecules secreted by these cells [225, 232, 233].

Bone marrow mesenchymal stem cells (bMSCs) are also widely used for bone tissue engineering because of their proliferation ability, high potential for osteogenic differentiation, and good osteogenesis ability. However, bMSCs are difficult to acquire due to histocompatibility of the donors, leading also to greater trauma when collected and to other clinical complications. To solve these problems, researchers have shifted their attention to other sources, with less invasive collection methods and collateral negative implications like human dental pulp stem cells (hDPSCs). Compared with bone marrow MSCs, hDPSCs are easy to obtain, resulting in a short trauma recovery process, considering that several individuals that are performing orthodontic treatments, sometimes need to remove healthy teeth. Moreover, the economic burden borne by patients is smaller, for these reasons, deciduous teeth, premolars extracted in orthodontic treatments and extracted third molars provide good hDPSC sources. Vascular cell adhesion protein 1 (VCAM-1), cluster of differentiation 146 (CD146), and alpha-smooth muscle actin (α-SMA), and pericyte and mesenchymal stem cell markers 3G5 ganglioside antigen and Stromal Cell Precursor Surface Antigen (STRO-1) are DPSCs markers from the perivascular niche [215, 229, 232-235]. DPSCs were isolated from both the perivascular and the sub-odontoblastic compartments, and present identical cell size, doubling times and karyotype stability, differing only in morphology with rounder cells in the sub - odontoblastic compartment versus spindle shaped cells with long processes in the perivascular one [236]. The assumption of multiple sources or niches for the multipotent DPSCs is further supported by *in situ* markers expression profiles. Besides the classically considered perivascular niche, cells with multiple lineage differentiation associated markers were identified intra - nerve, within the pulp connective tissue (pulpocytes) as well as in a perineural location, considering evidence that CD146 and α-SMA might not be putative markers for dental cells with stem cell characteristics [237].

Stem cells form permanent human teeth dental pulp

DPSCs are commonly isolated from pre-molars. Supernumerary teeth or a tooth extracted after a traumatic crown fractures are also able to be a source for populations with stem cell characteristics but an inflammatory environment may affect the isolated cells proliferation and differentiation capacities [238, 239]. hDPSCs are documented to be established with approximately 80% success rate from freshly extracted teeth. An immediate isolation seems not to be an essential pre-requisite to adequate DPSCs number, since satisfactory numbers can still be recovered after five days of tooth extraction, if kept in adequate refrigerated transport medium [240].

Heterogeneous populations of isolated DPSCs present differences concerning clonogenic, proliferation and differentiation capacities. The existence of at least two different morphologies within isolates cells is also observed: spindle shaped cells with long processes (associated with perivascular location) and more rounded cells (associated with the outer area of the pulp [218, 232, 236, 241, 242]. The presence of multiple stem cell populations within the tooth is attributed to its embryonic origins, as teeth originate from two distinct embryonic layers, ectomesenchyme of neural crest and ectoderm of dental lamina [234, 236]. Another important reason for the presence of multiple stem cell is the fact that enzymatically obtained isolated are not pure stem cell cultures, but rather contain different types of stem/progenitor cells, as well as more committed fibroblast-like cells [242].

DPSCs usually perform the asymmetric cell division, originating cells with different potentials, i.e. ones tend to retain a stem-cell fate and others tend to generate differentiated progenies. As such, serial passaging of DPSCs results in loss of self-renewal capabilities and the deviation to self-differentiation. As proliferation arrests in routine expansion medium, the expression of differentiation marker genes and proteins increases [Alkaline phosphatase (ALP), Osteopotin (OPN), Osterix (OSX), Bone sialoprotein (BSP), Collagen type II (Coll II), Dentin sialophosphoprotein (DSPP)] as does the response of those cells to osteoinducing medium. *In vivo*, implanted undifferentiated cells at late passages present tendency for bone production, with decreased dentin and cartilage production potential [243].

Phenotypic features

DPSCs generally present increased proliferation rates when compared to the 'gold standard' of MSCs populations (bone marrow MSCs or BM-MSCs). Gene expression analysis agrees with such observation, since among 4400 genes commonly expressed by DPSCs and BM-MSCs, Insulin-like growth factor 2 (IGF-2, cell growth stimulation properties) and Insulin-like growth factor-binding protein 7 (IGFBP-7, cell proliferation inhibitor) are up- and down-regulated in dental cells [244]. DPSCs high proliferation and population-doubling times are reported to be quite stable up until the 40th population-doubling (12-50 hours), increasing then to 60-90 hours. Their chromosomic stability and undifferentiated state is maintained beyond the Hayflicks limit (approximately 50 population doublings), for at least 60 population doublings [236].

These cells express common phenotypic markers with BM-MSCs, recognized for their stemness potential, while absent of major hematopoietic markers (CD14, CD34 and CD45) [215]. DPSCs cultures obtained in our laboratory according with other research groups, exhibited ≥ 95% expression of CD73, CD90, and CD105, with ≤ 5% expression of CD34, CD45, CD11b, CD19, and HLA-DR, as well as tri-lineage (osteogenic, adipogenic, and chondrogenic) differentiation capacity [240]. DPSCs are also reported positive for CD44 (Cell surface markers associated with stromal cells, like CD90), CD166 (Cell surface markers associated with stromal cells and endothelial cells, like CD105), CD146 (Cell surface marker associated with stromal cells, endothelial cells and perivascular cells), but negative for CD40, CD80, and CD86 (Cell surface markers of hematopoietic cells). HLA-ABC (MHC class I antigen) high expression is also in accordance to BM-MSCs [245].

Although DPSCs are classically compared to BM-MSCs, they also reveal close similarities with other emerging stem cell sources, such as the stromal tissue from the umbilical cord, named Wharton's jelly. Phenotyping revealed a similar expression pattern of antigens characteristic of MSCs including CD105, CD29, CD44, CD146, and STRO-1 on both cell types. DPSCs revealed to have less potential to adipogenic lineages, but were superior in terms of osteogenic and chondrogenic potential differentiation [246]. STRO-1 is a mesenchymal stem cell marker and its expression can be detected is about 9 to 23% of the isolated cells, with most of this fraction displaying colony-forming capacity. However, low expression STRO-1 populations do

display several stemness attributes and differentiation capacity. CD146 is deemed a perivascular marker, associated with DPSCs CFU capacity, although it appears not to be an essential requisite to multi-lineage differentiation [229, 242, 247-249]. In spite of being a reference marker for the classification of MSCs, the expression of CD34, expressed in primitive pluripotent stem cells, of both stromal and hematopoietic origin, the expression in DPSCs is controversial [215, 227, 240, 242, 248, 250]. Since some authors report $CD34^+$ DPSCs sorted isolates retaining all the remaining mesenchymal stem cell features and even enhanced *in vitro* and *in vivo* bone formation capacity [209, 233, 251]. CD34 is expressed by adherent dental pulp cells, representing about 10% of the population and it is regarded as a cell surface marker expressed by the most primitive stromal other than hematopoietic stem cells, before lineage commitment. Most often, $CD34^-$ cells were reported to differentiate into bone producing cells *in vivo*. An array of pluripotency/ embryonic stem cell markers can also be identified in DPSC isolates [212, 233, 249].

Differentiation capacities

Dental tissues provide cells having mesenchymal stem cell characteristics such as fibroblast-like structure (**Figure 17**), expression of surface antigens specific for mesenchymal stem cells, regeneration ability, multi-lineage differentiation capacity and immunomodulatory features. DPSCs, dental follicle progenitor cells, stem cells from apical papilla, tooth germ stem cells and periodontal ligament stem cells are stem cells derived from dental tissues as well as stem cells from exfoliated deciduous teeth. DPSCs have showed differentiation capacity towards multiple other mesodermal and endodermal lineages, under appropriate conditions: adipogenic, osteo/dentinogenic, chondrogenic, neurogenic, endothelial, myofibroblastic [237] and hepatocytes [249].

DPSCs differentiation potential is different to other possible sources of MSCs, for their apparently diminished ability towards adipogenesis and tendency towards mineral tissue formation [246], i.e. cells resulting from the first isolation procedures were shown to *in vivo* differentiated into adipocytes and odontoblasts and regenerate dentin-like tissues. As primary cultures already mark positive to bone-related markers and indications of spontaneous differentiation have been observed [215, 242]. DPSCs marker expression profile indicates a tendency of these towards the

neuroectodermal lineage differentiation with successful neuronal differentiation occurs [235, 252].

DPSCs from other species

Several studies of structural and functional regeneration of dental pulp have culminated with the isolation and characterization of DPSCs in different animal species such as dog, pig, sheep, mouse, rat, ferret, rabbit, cattle and chimpanzee. The best models in dental and orofacial investigation are considered larger animals, but small animals are often chosen due to costs, breeding and ethical issues [253, 254].

For instance, Murine DPSCs (mDPSCs) and Rat DPSCs (rDPSCs) have been successfully isolated and differentiated in several cell lineages [216, 231, 241, 255-263]. Juvenile and adult rat DPSCs display distinct proliferation and differentiation capacities, which can be modulated through the application of differently aged conditioned medium to the cultures [231]. *In vivo* they have also shown self-differentiation capacity, by forming bone, dentin and cartilage like tissues when implanted in rat renal capsules [243]. Rabbit dental stem cells (raDPSCs) were evaluated in scaffolds of different porosities for their potential to regenerate dentine/pulp tissue. In order to test the regeneration of critical-size alveolar bone defects using raDPSCs, ceramic/polymer composite materials and recombinant rhBMP-2, have been used as a preclinical animal model [264, 265]. A recent report describes the isolation and characterization of ferret DPSCs [266].

Other emerging and growing field of application is also the Veterinary cellular therapies using DPSCs, but further research concerning MSCs isolation, expansion and characterization from domestic and production animals is further needed. Cells from canine teeth have also been isolated (cDPSCs) and their full characterization has been attempted. These have been assessed for their regenerative capacity at the level of dentin, dental pulp and bone [253, 267-269]. Similarly, to human-derived DPSCs, cDPSCs also present the capacity to adhere to plastic surfaces and extensively proliferate and display fibroblast-like morphology, CFU formation and multi-lineage differentiation potential. However, phenotypical characterization remains challenging, due to the limited availability of species-specific antibodies [253].

Swine cells isolated from mini-pig incisor teeth also form adherent clonogenic cell clusters of fibroblast-like cell morphology, forming an heterogeneous spindle-shaped cell population, comprising of 10.9% STRO-1+ cells [270, 271]. Chimpanzees DPSCs, collected post-mortem, produced stem cell cultures remarkably similar to those of human BM-MSC and DPSCs. Chimpanzee's DPSCs expressed stem cell markers (Nanog, Sox-2, Rex-1 and Oct-4) and underwent multi-lineage differentiation (osteogenic, adipogenic and chondrogenic) [272]. Both DPSCs and PDLSCs have been isolated from ovine teeth. Both populations expressed markers CD44, CD106, and CD166 and lacked expression of the hematopoietic markers CD14 and CD45 and the endothelial marker CD31 [273, 274].

MSCs isolation

The isolation of MSCs populations from dental pulp is reported through three methods, either alone or combined according to of the dentition and species or origin: the simple mechanical dissociation, the explant culture and the enzymatic digestion. The mechanical dissociation method has been described to effectively isolate DPSCs, however explant culture and enzymatic digestion of the connective pulp tissue are by far the most widely applied methods [275, 276].

Explant isolation requires for an extended isolation period, since DPSCs take 7-15 days to initiate migration from the seeded tissue into the culture surface, where they then expand and quickly provide numerous cells and it is also possible to apply a pre-digestion step to the explant procedure [246, 277-280]. DPSCs with explant isolation can be largely increased by mechanically transferring the explant tissue to new culture surfaces every three or four days. The process can be extended for at least six months, continuously assuring early passage cells without jeopardizing cell morphology, expression of stem cells markers, chromosome stability, or differentiation potential, even after cryopreservation [281].

Enzymatic digestion can be achieved using a primarily type I collagenase, alone [227, 235, 282-288] or in combination to other dissociation agents, such as dispase [229, 236, 237] DNAse [232] or type II collagenase and thermolysin [216, 240, 289]. Although a comparative study of 10% FBS supplemented αMEM and DMEM for culture expansion suggest that the first is more adequate for DPSCs, the DPSCs do not seem to present specific culture requirements [262].

MSCs culture methods

Classic DPSCs culture medium contains serum from xenogenic origin, however, its use rises several key issues. It potentiates the occurrence of immune reactions when implanted (due to the presence of immunogens or toxic components) and increases the risk of pathogen transference of animal origin. Detailed composition of serum is still unknown and prone to relevant batch-to-batch variation. Moreover, it is difficult to use it for research and clinical application [283, 290]. Additionally, medium formulations containing serum may predispose to malignant transformation in latter population doublings [291].

Accordingly, low-serum and serum free formulations have been explored, for both isolation and expansion, as well as for differentiation protocols. Low-serum medium (2% foetal calf serum - FCS) supplemented with 10 ng/ml EGF (Epidermal growth factor) and 10 ng/ml PDGF-BB (Platelet derived growth factor-BB), originally formulated for the expansion of Mesenchymal progenitor cell from the Bone-Marrow, it is able to sustain the proliferation while preserving the stem cell phenotype of DPSCs and differentiation capacity into multiple lineages. The main differences may be found in terms of initial cell adhesion and CD146 and α-SMA markers expression that might be related to an initial selection on the adherent cells, imposed by the serum level. The addition of insulin-tranferrin-sodium selenite (ITS) supplement to the low-serum medium improves population doubling times [236, 291].

Is also possible the replacement of animal origin serum by human sources. Substitution of foetal bovine serum (FBS) content for human umbilical cord blood (UCB) plasma/serum, or derived platelet rich plasma (PRP) resulted in increased proliferation of DPSCs, SHEDs and PDLSCs [250]. Phenotypical profiles of mesenchymal and embryonic markers were maintained or even optimized and osteogenic differentiation capacity was unaffected in cells expanded in human serum containing medium [290]. Completely serum-free conditions appear to sustain acceptable 84%–90% viability (although significantly inferior to 90%–93% in 10% FBS medium) and stem cell markers expression was maintained [283]

Immunomodulatory properties

Immunomodulatory characteristics of DPSCs make them suitable candidates for the therapy of immune-related disorders. For that reason, DPSCs implantation in bone defects does not elicit a rejection response and DPSCs integrate and contribute to new tissue formation, allowing for allo-transplantation without host rejection [292].

DPSCs present potentially superior immunosuppressive/immunomodulatory properties comparing for instance to BM-MSCs [279]. The application of conditioned medium from stimulated or unstimulated DPSCs populations from dental tissues, results in differential immune responses suggesting that the inflammatory stimulation is also of utmost importance to the level and type of immunoactive soluble factors secretion [245]. So the immunosuppressive role of MSCs in general, may be modulated by the immune stimulation they suffer as well as by their tissue source [232, 245].

Cryopreservation of pulp tissues and DPSCs

Cryopreservation of pulp tissues and DPSCs is crucial for clinical application purposes and a few key parameters must be thoroughly considered: freezing medium composition, cell density, and freezing temperature. It is essential the adjustment of these parameters for optimal cell viability upon thawing, and for the preservation of their relevant features [240, 287].

The best cryoprotector is Dimethyl Sulfoxide (DMSO), and as for cell concentration, post thaw viability is maintained up to a concentration of $2x10^6$ cells/ml. For at least 6 months, the frozen vial can be maintained at -85°C or -169°C, with no major impact on cell viability, growth rate, specific marker levels or differentiation capacity even for long-time preservation until 2 years [251, 287, 293].

Cryopreservation of intact teeth without performing micro-channels is not a viable method, due to the poor freezing medium penetration, neither is the cryopreservation of pre-digested pulp tissue, due to cumulative cell damage from enzyme and freezing process on the cell membrane. Before preservation would be highly desirable a minimal manipulation of the tissues and it is possible to isolate viable DPSCs from cryopreserved teeth [240, 247, 289]. Alternatively, the creation of

micro-channels at the level of the crown-root connection enables the cryoprotector to effectively penetrate into the pulp tissue, enabling the recovery of comparable cell numbers to those obtained from fresh teeth, preserving proliferation and phenotype of the DPSCs [227].

DPSCS potential in tissue regeneration applications

DPSCS are able to undergo myogenic, chondrogenic, adipogenic, neurogenic, osteogenic and odontogenic differentiation ability and they can easily be manipulated in regenerative medicine. Cell-based strategies have emerged as promising treatment strategies when the intrinsic regenerative potential is diminished by age, health or other factors, concerning the bone tissue [39]. DPSCs are relatively abundant cells, easily to be recovered and demonstrated high differentiation ability. DPSCs are excellent candidates for therapeutic applications concerning the bone regeneration. [212, 214, 218, 294-298]. Many factors determine the success of cell-based therapies for bone defects, including: i) the type of cells and vehicle used to transport them; ii) final cell concentration; iii) immature or progenitor cell status; iv) when the therapy is applied; v) mode of administration and vi) availability of osteoblastic progenitors at the fracture site. For example, the availability of osteoblastic cells at the fracture site constitutes another critical factor, which can be manipulated. Overall, recruitment (i.e., homing) of endogenous MSCs to bone defect sites presents benefits and limitations when compared to exogenously administered autologous or allogeneic MSCs [299].

Bone tissue regeneration

Glycolic acid and lactic derived polymers are important biomaterials with interest in the tissue engineering field. Severe reasons are associated to this importance, such as biocompatible metabolites, controlled hydrolytic degradation and production of porous scaffolds may be achieved by different methods. Both synthetic and natural polymers, have been used in tissue engineering [300].

In terms of osteo-differentiation and mineralization of DPSCs, the poly-lactic-glycolic acid (PLGA) 85:15 presenting topographic microncave features demonstrated advantageous over convex HA coated titanium surfaces [301].

STRO-1 positively selected DPSCs were seeded onto Polycaprolactone (PCL)/ Gelatin electrospun meshes, with or without nano-hydroxyapatite (nHA). Both situations resulted in *in vivo* ectopic new bone-like hard tissues with entrapped osteocyte-like cells and osteoblast-like cells lying orderly arranged at the surface of the implants, but enhanced gene expression was observed on the nHA containing scaffolds [302].

Three types of materials comparably supported the attachment, growth and *in vivo* differentiation of human DPSCs *in vitro* in a study that evaluated the reaction of DPSC on three kinds of scaffolds materials: a spongeous collagen, a porous sintered HA/TCP, and a fibrous titanium mesh. The *in vivo* analysis demonstrated that cell loading impacted on the amount of newly formed tissue and on its vascularization. All the tested materials only little amounts of hard tissue formed following 12 weeks of *in vivo* implantation on immunocompromised mice [303].

DPSCs seeded onto a highly porous natural hydroxyapatite scaffold (Biocoral®) were able to migrate and adhere inside the structure and differentiate and produce osseous matrix. Hydroxyapatite based material are a recurrent choice for mineralized tissue engineering and it is able to produced matrix completely integrated with the scaffold. By the differentiating cells, osteoblast-related gene expression (OC, OPN and BSP) was highly up-regulated in mineralized tissues. Also culture medium analyses revealed enhanced bone- and angiogenic-related growth factor production (Bone morphogenic protein 2 (BMP-2) and vascular endothelial growth factor (VEGF)) production [293].

In critical size calvarial defects (2.7 mm diameter) an identical formulation implanted resulted in long term formation of mineralized tissue, and the newly formed tissue derived from SHEDs' effects on recruitment and induction of host cells, as well as from their own osteogenic differentiation and matrix production. *Ex vivo* expanded-SHED seeded onto HA/TCP carriers were implanted in the same model and developed comparable amounts of mineralized tissue to BM-MSCs. However, reports state that SHED-mediated bone lacked hematopoietic marrow elements, unlike BM-MSCs derived bone tissue [304-306].

Rat DPSCs on HA/TCP porous structures reciprocally are reported to give rise bone marrow-like hematopoietic tissue, within distinct concentric lamellae with

entrapped osteocyte-like cells and osteoblast-like cells, after subcutaneous implantation on immunocompromised mice [307].

APDCs seeded on similar structures and cultured in osteoinducing medium (with BMP-2) also displayed good cell adhesion and dissemination through the structure, and progressive increment in ALP activity. When implanted *in vivo* (subcutaneously in nude rat), bone-like tissue was patent after 3 weeks, containing osteocyte-like cells embedded within the calcified matrix, and osteoblast-like cells along the lining of the bone surface [308].

In rabbits, during reconstitution of alveolar bone defects was achieve with nano-hidroxiapatite/collagen/poli-L-lactic (nHAC/PLA) acid scaffold and recombinant human BMP-2. The complex nHAC/PLA with BMP-2 showed support, proliferation and differentiation ability towards DPSC representing a viable alternative to autologous bone transplantation in the bone tissue regeneration [265].

Autologous DPSCs have successfully restored critical size mandibular defects in a swine model. The seeded cells on a β-TCP scaffold provided nearly complete regeneration of initial defect sites (83% of the defect), which were filled with newly formed lamellar bone and degraded β-TCP scaffold and they were shown to remain in the implant, differentiate and actively contribute to bone formation. Cell loaded scaffolds resulted in improved regeneration when compared to the scaffold alone, with moderate to minimal bone formation within the defect, which was mainly filled by abundant un-mineralized connective tissue in the latter case [271].

In the last few decades the osteogenic DPSCs potential have been compared and *in vitro* DPSCs proliferation, differentiation and attachment to β-TCP, freeze-dried bone allograft (FDBA), and deproteinized bovine bone mineral (DBBM) has been demonstrated. As a matter of fact, these are the most common bone substitutes in clinical applications. β-TCP showed highest proliferation, while FDBA induced more significate osteoblastic differentiation [309-311] [312].

To evaluate the effectiveness of HA/TCP/chitosan scaffolds for osteogenic regeneration in mandibular defects were transplanted DPSC enriched scaffolds into mandibular defects in a male *Macaca nemestrina*, and results in an active bone and blood vessel formation [313].

ECM is largely comprised by fibrillary proteins that *in vivo* surround cells in a well-organized manner providing physical support and enabling cell adhesion and migration. Softer material like hydrogels and natural polymer derived mesh structures intent to mimic ECM characteristic have also been reported. Peptide-amphiphile can also be modified to enhance mineral deposition [314], ectopic bone formation [315] and to optimize cell adhesion [316]. The amphiphilic material self-assembles in aqueous environment into three-dimensional structures forming a gel matrix [317, 318].

Several studies showed that cells are able to move, proliferate, differentiate and grow within a hydrogel matrix. Comparing the mineralization of SHEDs and DPSCs on self-assembling bioegradable peptide amphiphile hydrogel, the first ones showed a tendency for soft tissue formation, on the other hand DPSCs developed more maturely mineralized tissues. SHED displayed enhanced ECM molecules expression (Coll I and Col III), especially in β-glycerophosphate/ dexamethasone medium, with only limited mineralization observed in KH_2PO_4/ dexamethasone medium, while DPSCs seemed more prone to osteogenic differentiation, with increased mineralization indicators in on potassium dihydrogen phosphate (KH_2PO_4)/ dexamethasone medium, suggested a further advanced lineage commitment [319].

Silk fibroin scaffolds with osteogenically driven human DPSC successfully regenerated calvarial critical defects. Implanted cells were still patent, in osteocyte and osteoblast typical location within the forming tissue, suggesting complete differentiation and a critical role for the effective bone regeneration [320].

Another treatment strategy within this area is the use of collagen membranes loaded with autologous expanded DPSCs and clinically applied to patients undergoing bilateral third molar extractions. In a human study using a collagen sponge and DPSCs in oro-maxillo-facial (OMF) bone tissue repair after bone defects treatment, revealed a complete re-sorption of the collagen sponges, well organized and well vascularized bone with a lamellar architecture surrounding the Haversian channels in the treated sites while control sites had a more immature bone, with fibrous bone entrapped among new lamellae, incomplete and large Haversian channels and evidence of bone reabsorption. Although all samples expressed bone proteins (like on ON and OC) a different distribution was noted, as well as increased expression of BMP-2 and VEGF in the DPSCs treated sites. Collagen type I and II scaffolds they were able to support

the *in vivo* differentiation of DPSCs into the osteogenic lineage. Histological and immunohistochemically results showed significate expression of ALP, BSP and OPN in transplants DPSCs in collagen scaffolds [321, 322].

Back to the immunocompromised mice model, silk fibroin scaffolds with osteogenically driven human DPSC successfully regenerated calvarial critical defects. At 30 days post implantation the treated defects were vascularized and filled with bone tissue. The internal and external tables were almost completely reconstructed and areas of spongy bone evidenced between them, intersperse with resorbing fibroin remnants. Implanted cells were still patent, in osteocyte and osteoblast typical location within the forming tissue, suggesting complete differentiation and a critical role for the effective bone regeneration [320].

Self-assembling nano-fiber hydrogels can also successfully support mineralized tissue formation. Subcutaneous implantation of those scaffolds resulted in vascularized mineral tissue formation, expressing both collagenous (Coll I) and non-collagenous (OPN, OC and osteonectin - ON) bone matrix proteins, depicting different stages of bone formation [285].

A limiting factor for the success of many bone regenerations attempted strategies is the vascularization of the implants. Complementary development of mineralized tissue and respective blood vessel supply are essential. Selected DPSCs populations (CD117+/ CD34+/flk-1+/ STRO-1+) give rise to two distinctive progenitor populations, in high serum osteoinducing medium. As cells differentiate, they lose their stem cell phenotype (CD117, CD34, CD90, CD133) and acquire osteoblastic (VEGFR-2+/ STRO-1-/ CD44+/ RUNX-2+, 70% of the population) and endothelial phenotypes (VEGFR-2+/ STRO-1+/ CD44+/ CD45+, 30% of the population). *In vitro* spontaneously formed bone chips displayed blood vessel structures within the mineralized matrix and in vivo implantation of those resulted in mature vascularized bone. Both osteocytes and endotheliocytes were mainly of human origin, containing host blood cells [323].

In canine model treated with MSCs, DPSCs and DT-MSCs with PRP was assessed as regenerative alternative in mandibular bone defects given the recognized importance of growth factor delivery and integration into regenerative systems. After four weeks it was detectable new bone formation (active osteocytes) in the presence of DPSCs. Then after eight weeks, mature bone structures with lamellar tissue and

abundant medullar cavity. Osteogenic supplementation was employed with concomitant addition of PDGF, Transforming growth factor beta (TGF-β) and IGF-1. The naturally formed scaffold was able to promote bone tissue regeneration as it was previously described with MSCs [268].

Chapter 5 – *In vitro* evaluation of Bonelike®

Introduction

This chapter presents the tests performed with the objective of evaluate the *in vitro* biological behaviour of the Bonelike® samples in the presence of hDPSCs in terms of cell adhesion to the biomaterial, cell viability/proliferation and evaluation of the ability of the biomaterial to support the osteogenic differentiation of hDPSCs (osteointegration of the biomaterial). These tests were developed in the Laboratory of Veterinary Cellular Therapies of ICBAS-UP.

Materials and methods

In vitro validation – celular viability evaluation

Experimental design

In this experiment three tests were performed according to ISO 10993-5. This norm "Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity" defines and describes test methods to evaluate the *in vitro* cytotoxicity of medical devices**.** These methods are designed to determine the biological response of mammalian cells *in vitro* using the appropriate biological parameters (**Table 3**).

Table 3: Experimental design of the tests performed.

No Test	Bonelike® Fixing Agent	Type of test	Tests performed			
			Presto Blue	Inverted Optical Microscope with Phase Contrast	SEM	Osteo differentiation
1	Agar-Agar (4 % agarose gel) 	Direct	X	X	X	
2	Cytocompatible Glue 	Direct	X	X		
3	*Insert* 	Indirect / by extraction	X	X		X

Sample preparation

Bonelike® was previously steam sterilized in an autoclave (Uniclave® 88, Portugal) for 20 minutes at 121°C and 15 psi pressure.

Cell cultures and maintenance

Human DPSCs (hDPSCs) were obtained from AllCells®, LLC (Cat. DP0037F, Lot No. DPSC090411-01). hDPSCs were prepared and seeded at a density of $7x10^3$ cells / cm^2 in a 24-well plate and maintained in α-MEM, GlutaMAX™ Supplement, without nucleosides (Gibco®, 32561029) supplemented with 10% (v / v) fetal bovine serum

(Gibco® A31608-01), 100 IU / mL penicillin, 0.1 mg / mL streptomycin (Gibco®, 15140122), 2.05 μg / mL amphotericin B (Gibco®, 15290026) and 10 mM HEPES buffer solution (Gibco®, 15630122). Cells were maintained at 37°C in a humid atmosphere with 95% atmospheric air and 5% CO_2.

Assay for cell viability using PrestoBlue™

In the cell viability / cytotoxicity tests, the PrestoBlue™ reagent was used. This reagent is a resazurin (7-hydroxy-3H-phenoxazine-3-one-10-oxide) solution commercially available, ready to use and water soluble. Living cells reduce this compound in resafurin, a process that is accompanied by the colour change and fluorescence of the solution. Thus, the absorbance measures of the solution function as an indicator of cell viability that allow quantitative measurement of cell proliferation. Due to the reduction of the compound by the viable cells, the coloration of the solution changes from blue to reddish. Thus, changes in cell viability / proliferation can be assessed by the corresponding changes in absorbance measurements.

The Bonelike® granules were fixed on glass slides and placed at the bottom of each well of a 24-well plate, and the relevant controls were prepared with the fixative alone and the granules without the cells. The granules were seeded at the concentration of $10x10^3$ hDPSCs / well density. Cells were adhered in 0.5 ml of complete culture medium overnight.

At each time point [24 hours (1 day), 72 hours (3 days), 120 hours (5 days) and 168 hours (7 days)], the culture medium was removed from each well and fresh complete medium 10% (v / v) of 10 times the cell viability reagent, PrestoBlue™ (Invitrogen®, A13262) was added to each well.

The cells were incubated for 1 hour at 37°C in 5% CO_2. Changes in cell viability were detected by absorbance spectroscopy in the spectrophotometer, Thermo Scientific Multiskan FC. The supernatant was collected and transferred to a 96-well plate and the absorbance read at 570nm and 595nm. For each well, the absorbance at 595 nm (normalization wavelength) was subtracted from the absorbance at 570 nm (experimental result). The corrected absorbance is obtained by subtracting the mean of the control wells for each experimental well.

The cells were then washed in Dulbecco's phosphate buffer (DPBS, Sigma®, D8537) to remove any PrestoBlue™ residues and then the fresh culture medium is replenished to each well.

Calculation of percentage reduction of PrestoBlueTM reagent

To analyse the data obtained, the absorbance values are converted to a percentage reduction of the PrestoBlue™ reagent using the molar extinction coefficients of the oxidized and reduced forms of this reagent. The percentage reduction of the PrestoBlue ™ reagent can be calculated from the absorbance data using the following equation (Equation_2):

$$\text{Percentagem de redução do Reagente } \textit{PrestoBlue}™ = \frac{(O2 \times A1) - (O1 \times A2) \times 100}{(R1 \times N2) - (R2 \times N1)} \qquad \text{[Equação_2]}$$

Where:

O1 = molar extinction coefficient of the oxidized PrestoBlue™ reagent at 570 nm = 80586

O2 = molar extinction coefficient of the oxidized PrestoBlue™ reagent at 600 nm = 117216

R1 = PrestoBlue™ reagent molar extinction coefficient reduced to 570 nm = 155677

R2 = PrestoBlue™ reagent molar extinction coefficient reduced to 600 nm = 14652

A1 = absorbance of the test wells at 570 nm

A2 = absorbance of the test wells at 600 nm

N1 = absorbance of the mean of the wells only at 570 nm

N2 = absorbance of the mean of the wells only at 600 nm

By replacing the values in the equation, it is possible to obtain the graphical representation of the percentage of reduction of the PrestoBlue™ Reagent in each group of samples.

In vitro validation - Qualitative and semi-quantitative evaluation of osteogenic differentiation

Cell culture and differentiation

The hDPSCs were prepared and seeded at a density of 1.2 x 10^4 cells / cm^2 in a 24-well plate similar to the procedure described in 3.1.1.3. Cells were expanded for 48 hours and then transferred to a specific differentiation medium, the Osteogenesis (OM) media. The osteo-differentiation medium was formulated according to Kyllönen et al., Containing 5nM Dexamethasone (D8893 - Sigma Aldrich®), 250 µM ascorbic acid 2-phosphate (AsA2-P) and 10 mM Beta-glycerophosphate (β-GP) [86]. Control wells were maintained in standard culture medium. The media were replaced every 2 days for 14 days.

Alizarin Red S Test

Cellular viability / cytotoxicity tests were carried out using the Alizarin Red S solution (ARS) (TMS-008-C). This is used in biochemical assays to determine, qualitatively and semi-quantitatively, by colorimetry, the presence of calcium deposits from cells in an osteogenic cell line. This is a marker of matrix mineralization, a crucial step in the formation of calcified extracellular matrix associated with bone. This mineralization assay is more advantageous compared to other established calcium assays which have very limited linear detection ranges and do not allow direct visualization of the mineral prior to processing and measurement. This assay has a wide range of potential uses, including tumour characterization, evaluation of mesenchymal stem cells and screening for osteogenic compounds. This procedure was based on the work of Gregory *et al*. [324].

Alizarin Red S Stain

In each well of the cell culture dish, the culture medium was removed and washed 3 times with PBS. Cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature. After removing the fixative, the cells were washed 3 times with distilled water. 500 µl of 40 mM ARS were then added to each well. The plate was incubated at room temperature for 20 to 30 minutes with gentle shaking. After the dye was removed, the cells were washed 5 times with distilled water. The plate was

leaned for 2 minutes to facilitate removal of excess water. Subsequently, the plates were stored at -20° C.

Quantitative analysis of osteo-differentiation

To each well of the cell culture dish was added 200 µl of 10% acetic acid. The plates were then incubated at room temperature for 30 minutes with stirring. Cells were harvested with a cell scraper and transferred with acetic acid to a 1.5 ml microcentrifuge tube. Each tube was vortexed for 30 seconds and heated exactly at 85 ° C for 10 minutes. The tubes were then incubated on ice for 5 minutes and then centrifuged at 20,000g for 15 minutes.

After centrifugation, 100 µl of the supernatant from each well was transferred to a 96-well plate. The absorbance was read at 405nm. A standard ARS curve was then made using Excel and the ARS concentration in the samples was calculated by interpolation in the calibration curve. The ARS standards were prepared in successive dilutions from the 40nM ARS solution in 10% acetic acid. The concentrations of the prepared standards were: 4mM, 2mM, 0.5mM, 0.125mM and 0.0313mM, and the blank was 10% acetic acid. The solutions were prepared in triplicate, and their absorbance was analysed at 405nm.

All samples, standard and white solutions were analysed on the UV-VIS spectrophotometer and its absorbance at 405nm recorded. The absorbance was corrected by subtracting the mean blank value. The calibration curve was plotted based on the absorbance value of the standard solutions of ARS (absorbance at 405 nm) as a function of the ARS concentration). The equation of the line was determined and thus the sample concentrations were calculated.

Statistical analysis of the cellular viability evaluation

Statistical analysis was performed using GraphPad Prism version 6.00 for Mac OS X, GraphPad Software, La Jolla California, USA. The assays were performed in quadruplicate and the results were presented as Mean ± Standard Deviation (SE). The analysis was performed by the unidirectional ANOVA test followed by the Tukey multiple comparisons test. The differences were considered statistically significant at P0.05.

The meaning of the results is presented in comparison with the controls and represented by the symbol (*). The meanings of the results are also indicated according to the values of P (probability of significance) with one, two, three or four of the symbols (*) corresponding to $0.01<P<0.05$, $0.001<P<0.01$, $0.0001<P<0.001$ and $P<0.0001$, respectively.

Analysis by scanning electron microscopy

After evaluating cell viability, the Bonelike® samples were collected and washed three times with DPBS. Cells were fixed in 4% paraformaldehyde for 10 minutes. Subsequently, the samples were dehydrated in solutions with increasing concentrations of ethanol. The samples were coated with a thin film of Gold / Palladium by sputtering using the SPI Module Sputter Coater. The SEM / EDS test was performed using the high resolution (Schottky) Scanning Electron Microscope, with X-ray microanalysis and diffraction patterns analysis of the electrodes: Quanta 400FEG ESEM / EDAX Genesis X4M in high vacuum mode. Each image has the most important analysis conditions entered.

Results and discussion

In vitro validation – Evaluation of cell viability of hDPSCs

Direct contact test – Fixing medium: Agar-Agar

This assay was divided into three groups: Bonelike (plus agarose gel and culture medium with hDPSCs), agarose gel (plus culture medium with hDPSCs) and culture medium in the presence of hDPSCs, in quadruplicate. For each group controls were performed, without cells, in duplicate. Microscopic images relating to the assay in which the fixation medium used was Agar-Agar (4% agarose gel) is shown in **Figure 18.**

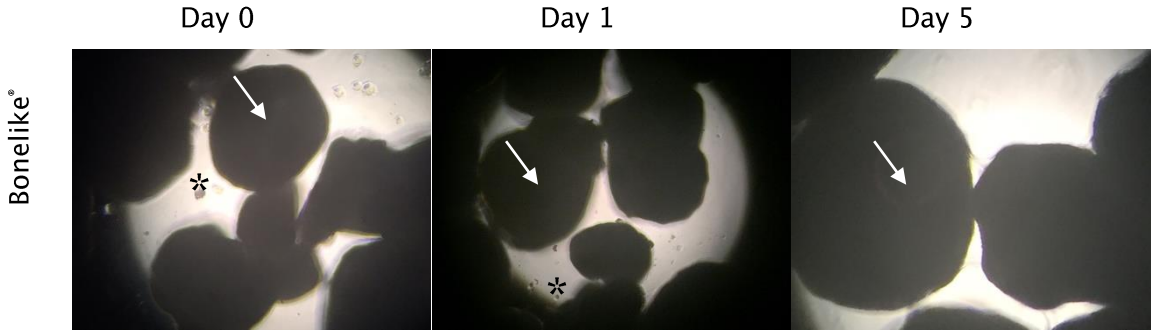


Figure 18 – Qualitative analysis of the assay with the agar-Agar fixative (4% agarose gel). Images obtained through the inverted optical microscope with phase contrast and are representative of the experimental groups with Bonelike ® (plus agarose gel and culture medium with hDPSCs). The images were obtained after 0, 1 and 5 days of cell culture of the hDPSCs (Total magnification: 100x). Arrows – biomaterial; asterisks – hDPSCs.

With the cell viability / cytotoxicity assay with the PrestoBlue™ reagent the Bonelike®, the gel and the media were compared after day 1, 3, 5, 7 and 10 of the hDPSCs cell culture (**Figure 19**).

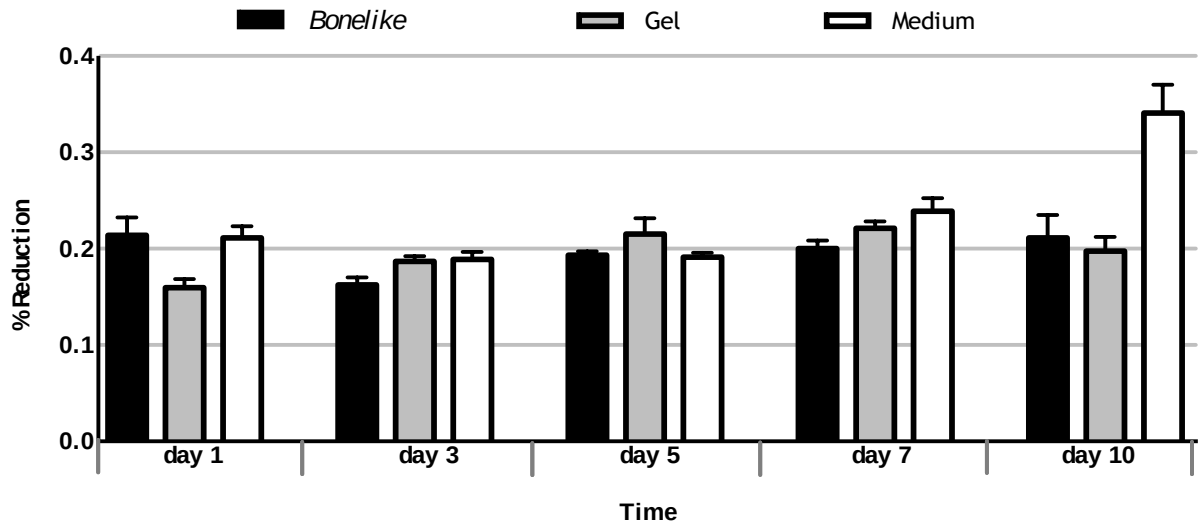


Figure 19: Quantitative analysis on agar–Agar binding agent assay (4% agarose gel). Results with mean and standard deviation of the % reduction of PrestoBlue™ in the experimental groups: Bonelike ® (plus agarose gel and culture medium with hDPSCs), agarose gel (plus the culture medium with hDPSCs) and culture medium in the presence of hDPSCs. The results were obtained after 1, 3, 5, 7 and 10 days of cell culture of hDPSCs. The % reduction is proportional to the number of viable hDPSCs in culture and is associated with Equation_2.

Through the results of the microscopic images (**Figure 18**) and the PrestoBlue™ (**Figure 19**) it can be seen that the cell proliferation of hDPSCs in Bonelike® and gel agarose did not occur over time. Only in the control without the fixative (agarose gel) is it possible to observe cell proliferation throughout the assay (up to day 10 of the assay). These results seem to indicate that the presence of the agarose gel affects proliferation, since proliferation in the control without the agarose gel is visible.

However, the presence of Bonelike® seems to favor cell proliferation, since at all times analyzed, the viability is superior compared to gel control.

In order to clarify whether the inhibitory effect on cell viability and proliferation is due to the fixing agent or to Bonelike, another fixation medium was used, which involves the direct contact of both Bonelike formulations with the hDPSCs.

Direct contact assay

Fixing medium: Cytocompatible glue

This assay was divided into three groups, Bonelike® (plus glue and culture medium with hDPSCs), cytocompatible glue (plus culture medium with hDPSCs) and medium in the presence of hDPSCs, in quadruplicate. Controls, without hDPSCs, were made in duplicate for each group. The results of the cell viability / cytotoxicity assays with PrestoBlue™ relative to the assay in which the fixation medium used was the cytocompatible glue are depicted in **Figure 20**.

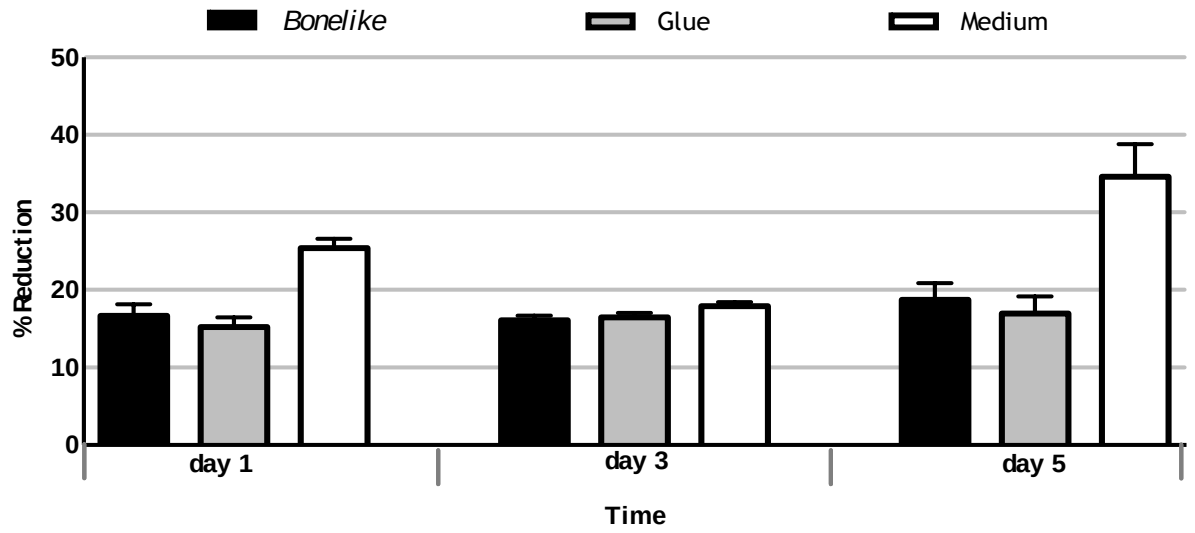


Figure 20: Quantitative analysis on the assay with the cytocompatible glue fixative. Results with the mean and standard deviation representation of the% reduction of PrestoBlue™ in the experimental groups: Bonelike® (plus glue and culture medium with hDPSCs), glue (plus culture medium with hDPSCs), and culture medium in the presence of hDPSCs. The results were obtained after 1, 3, 5, 7 and 10 days of cell culture of the hDPSCs. The % reduction is proportional to the number of viable hDPSCs in culture and is associated with Equation_2.

Through the results of PrestoBlue™ (**Figure 20**) it can be seen that no cell proliferation occurred in the Bonelike® containing wells and in control with the cytocompatible glue. Only in the medium without the fixative is it possible to observe cell proliferation throughout the assay. These results seem to indicate that the presence of this fixation medium affects proliferation, since proliferation in the control without the cytocompatible glue is visible, it was also verified the necessity of a large amount of glue to guarantee the fixation of the Bonelike® which may have influenced the results.

Since there was no cell adhesion and proliferation, the assay was discontinued. In order to conclude that the inhibitory effect occurred due to the fixation method, Bonelike® indirect contact with the cells was performed through inserts, as referred to in ISO 10993-5, point 8.3.

In this way, it is possible to ensure that the attachment medium does not interfere with cell viability and proliferation.

Indirect contact test

Fastening medium: Inserts

This assay was performed with the hDPSCs in indirect contact with Bonelike® granules. The hDPSCs were separated from the beads by a 70 μm membrane (in inserts). This assay was divided into two groups: one in which the Bonelike® within the inserts was soon placed in the well with the culture medium and hDPSCs (called "immediate") and another in which the Bonelike® within the inserts with the Bonelike® only were placed in the wells with culture medium and hDPSCs after 6 to 8 hours (called "delayed").

This division was made with the objective of observing if the inhibitory cellular activity that was observed in the previous tests was due to Bonelike® or the fixation medium used. Each was further subdivided into Bonelike® (plus culture medium with hDPSCs) and culture medium with hDPSCs. Controls were performed for each group without hDPSCs.

The results of the cell viability / cytotoxicity tests with PrestoBlue™ relative to the assay in which the fixation medium used was the cytocompatible glue are represented in **Figure 21**.

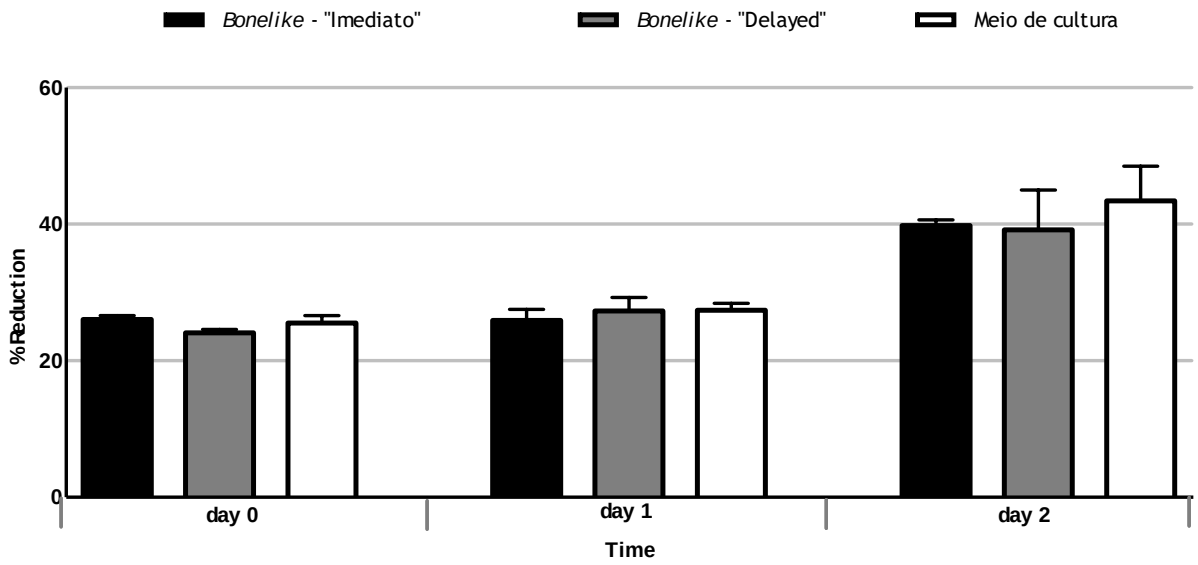


Figure 21 – Quantitative analysis in the assay where the fixative is the inserts. Results with the mean and standard deviation representation of the % reduction of PrestoBlue™ in the experimental groups: Bonelike® within the inserts (plus the culture medium with hDPSCs) and Culture medium with the hDPSCs. The results with Bonelike ® were further divided into 'immediate' and 'delayed' according to the time in which they were placed in contact with the culture medium and the hDPSCs (0 hours or 6 to 8 hours respectively). The results were obtained after 0, 1 and 2 days of cell culture of the hDPSCs. The % reduction is proportional to the number of viable hDPSCs in culture and is associated with Equation_2.

By analyzing the results (**Figure 21**) it is apparent that cell proliferation was very similar in all cases: Bonelike® "immediate" and "delayed" and "delayed" and in control. This reveals that the presence of Bonelike® did not affect cell adhesion.

Also, the fact that the control has similar results to those shown in the other samples leads to the opinion that the inhibitory factor for proliferation was the medium of fixation, not the contact with Bonelike® and substances that it can release.

This test was thus extended until the 9th day, in order to evaluate cell proliferation (**Figure 22**).

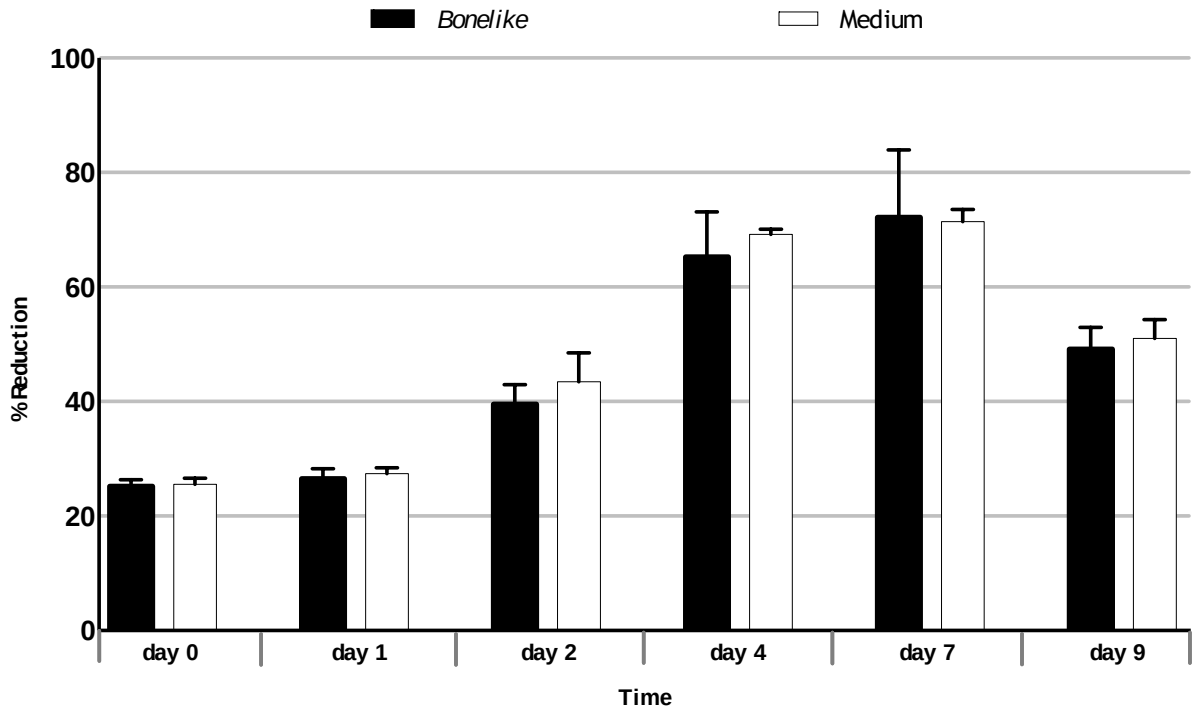


Figure 22- Quantitative analysis in the assay where the fixative is the inserts. Results with the mean and standard deviation representation of the % reduction of PrestoBlue™ in the experimental groups: Bonelike® within the inserts (plus the culture medium with hDPSCs) and Culture medium in the presence of hDPSCs. The results were obtained after 0, 1, 2, 4, 7 and 9 days of cell culture of hDPSCs. The % reduction is proportional to the number of viable hDPSCs in culture and is associated with Equation_2.

The analysis of **Figure 22** reveals that cell proliferation occurred in all samples over the first 7 days. This result confirms that the contact of hDPSCs with Bonelike® and substances that it can release does not influence cell viability. The results on day 9 would already be expected, since the cells are in the phase of decline / apoptosis.

The statistical analysis performed between the Bonelike® samples and the controls showed that the difference in values was not significant for $P \leq 0.05$ ($N = 7$). After evaluating the in vitro biological behavior of the Bonelike® samples, the granules were found to provide cell viability / proliferation. It has also been shown that the inhibitory effect on cell viability and proliferation was due to the Agar-Agar fixation agent and cytocompatible glue.

In vitro validation

Osteo-differentiation of hDPSCs in the presence of the biomaterial

This test was performed with the objective of evaluating the ability of biomaterials to support / promote osteogenic differentiation (osteo-differentiation in the presence of the biomaterial). The qualitative analysis (**Figure 23**) shows Bonelike® (plus OM with hDPSCs) and OM with hDPSCs (in quadruplicate) with the respective controls (in triplicate) in contact with Alzarin Red S. Bonelike® was in contact with the hDPSCs through inserts for 14 days.

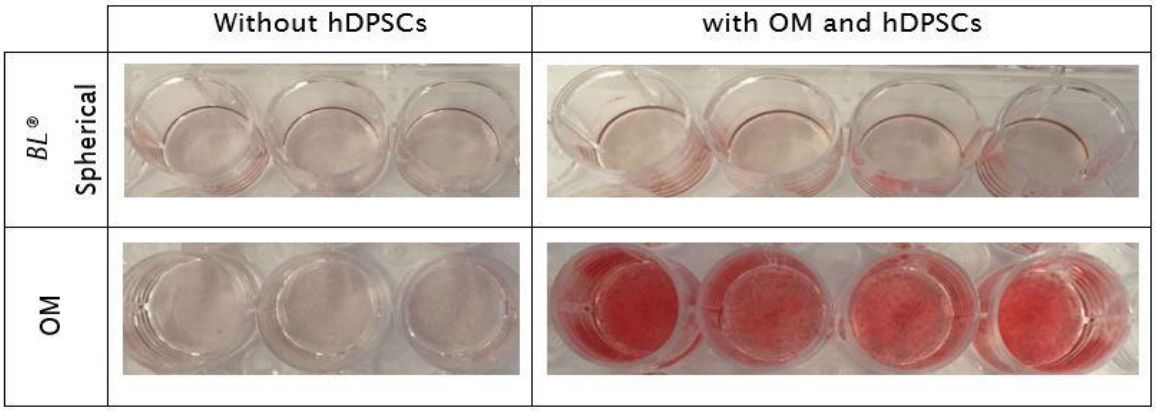


Figure 23 – Qualitative analysis of Alzarin Red S with inserts as fixation agent. Representative images of the quadruplicate experimental groups: Bonelike® within inserts (plus OM with hDPSCs) and OM medium (with hDPSCs). Each group also presents the respective controls, without hDPSCs, in triplicate. The images were obtained after 14 days of cell culture of hDPSCs.

Qualitative analysis (**Figure 23**) demonstrates that Bonelike® granules appear to inhibit osteo-differentiation, since the amount of staining is significantly lower than that observed in the control (OM – only cells). The result of the semi-quantitative evaluation of osteo-differentiation of hDPSCs in the presence of Bonelike® is shown in **Figure 24**.

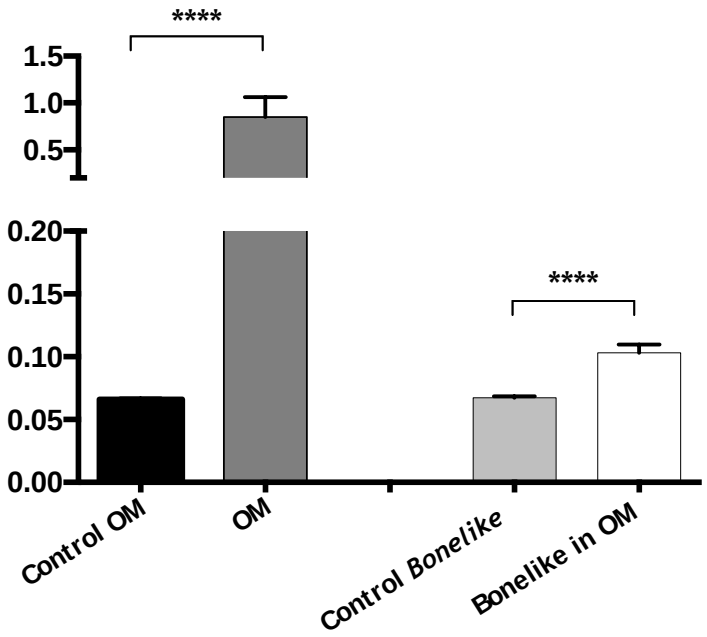


Figure 24 – Semi-quantitative analysis of Alzarin Red S with the representation of the mean and standard deviation of the experimental groups: OM with hDPSCs and Bonelike® (plus OM with hDPSCs) with the respective controls, without OM, after 14 days (****, P≤0.0001).

Through the semi-quantitative analysis (**Figure 24**), all samples in the presence of the osteo-differentiation medium showed a significant increase in the amount of calcium when compared to the respective controls after 14 days. With these results, cell proliferation and differentiation of hDPSCs are visible.

However, considering the average of the wells with the cells in OM is quite superior compared to those with Bonelike® in OM. The study by Lopes *et al.* reports that the chemical, topographical and electrical characteristics of the surface of the biomaterials have a pronounced influence on the growth and function of the cells that are in contact with these surfaces. The Bonelike® contains in its composition β-TCP that has a higher dissolution rate than the hydroxyapatite, thus being expected to have higher leaching of ions on the surface of the biomaterial. This is likely to cause changes in pH and probably modulate a series of functional activities of the cells.

However, such leachate substances may not necessarily be harmful. The specific design of these materials may have clinical value in the active promotion of processes of regeneration of bone and connective tissue after dental and orthopedic surgery.

Although in the above cytocompatibility assays, a significant inhibition of viability up to 7 days of incubation is not visible, longer incubation times (14 days of incubation) are assumed in this osteo-differentiation assay. Thus, there is the possibility that the release of ionic species more pronounced at later times of incubation has occurred, thus affecting the ability of cell differentiation. Being a closed and small volume medium, it does not replicate what happens in vivo, where there is an open physiological environment and there are no changes in pH.

Conclusions

In this chapter, assays were performed with the objective of evaluating the Bonelike® samples *in vitro* in the presence of hDPSCs, thus evaluating cell adhesion to the material, cell viability / proliferation and the ability of the materials to support / promote osteogenic differentiation of hDPSCs.

Adhesion and cell viability were assessed by two direct assays using agarose gel and cytocompatible glue as a fixative of the Bonelike® granules, as well as an indirect assay using inserts with Bonelike® beads. However, through the tests carried out, it was concluded that the presence of the agarose gel and the cytocompatible glue as a fixation medium affected adhesion and cell viability / proliferation and therefore, it was not possible to evaluate the cellular adhesion to the material.

After evaluation of the biological behavior *in vitro* with the inserts as Bonelike® fixation medium, the granules were found to provide cell viability / proliferation and therefore are not cytotoxic.

The ability of Bonelike® to support cell differentiation was assessed, with the inserts as a fixation medium. Considering the mean of the wells of the control with cells in OM this is much higher compared to the wells with Bonelike® in OM. The study by Lopes et al. reports that the chemical, topographical and electrical characteristics of the surface of the biomaterials have a pronounced influence on the growth and function of the cells that are in contact with these surfaces.

The Bonelike® contains in its composition β-TCP that has a higher dissolution rate than the hydroxyapatite, thus being expected a higher leaching of ions on the

surface of the biomaterial. This is likely to cause changes in pH and, modulate a series of functional activities of the cells. Thus, there is the possibility of the more marked release of ionic species at the later times of incubation, thus affecting the cell differentiation ability.

However, *in vitro* assays are performed in closed and small volume media, thus not replicating what happens *in vivo*. For this reason, *in vivo* assays are so important to more accurately represent conditions in the human body. Although the *in vitro* tests are an easy, fast, reproducible, sensitive and financially accessible tool, the tests performed reveal the importance of *in vivo* tests, since they are more reliable and reproduce real conditions, such as the conditions of the medium and the variations of pH.

Throughout the *in vitro* tests, some difficulties were presented in the manipulation of the granules, since the material has enough static electricity and some hydrophobia. This is due to the presence of the secondary α-TCP phase that influences the surface charge and the hydrophobicity of Bonelike®.

In the future, it would be important to use a direct fixation medium that allows in vitro evaluation of cell adhesion, viability and proliferation, without the inhibitory effect of Bonelike® fixation media being detected on the bottom of the cell culture plate.

Chapter 6 – *In vivo* evaluation of Bonelike® in non-critical defects associated to a cell-based therapy – hDPSCs

Introduction

Before biomaterials are applied to non-critical defects, several studies were performed to evaluate the safety of their use in terms of cytocompatibility, cell adhesion, biocompatibility, osteoconduction, osteointegration, and osteoinduction. In order to evaluate these parameters, both *in vitro* and *in vivo* tests are always necessary. *In vitro* tests do not completely replicate the behaviour and reactions of living tissues, so an *in vivo* evaluation of biomaterials and the associated cellular system, is required in adequate animal models, which can often raise some ethical issues [325].

Selection of the animal model

In the field of orthopaedics, translational research of novel therapeutic approaches involves the use of large animal models (such as sheep, goat, pig, dog, and horse) due to the similarities with humans in weight, size, joint structure, and bone/cartilage healing mechanisms [326].

When deciding which animal model is used for a particular study, there are several factors that should be considered such as: sex, age, weight, race and environmental conditions. Other points to consider are the acquisition cost, maintenance, ease of handling and housing of animals, ethical issues, ecological consequences of using the chosen animal model, resistance to infection and diseases, tolerance to surgery and a database of biological information for the species. In addition, the shelf life of the species chosen should be adequate for the duration of the study, as well as its phylogenetic proximity to humans [132].

In studies investigating bone-implant interactions, an understanding of the species' specific bone characteristics is required, such as microstructure, bone composition, modelling properties and bone remodelling, as well as the biomechanical forces to which they are subjected. The size of the animal should be

considered to ensure that it is appropriate for the number and size of the implants chosen [325].

Ovine Model

The sheep model was selected because of its similarity to humans in body size and mechanical characteristics of the musculoskeletal systems. An adult sheep has a similar body weight of a human being and has long bones of adequate size for the implantation of human implants/prostheses, which is not possible in smaller species. It is also an animal model more ethically accepted by the scientific and medical communities. Another similarity is the presence of Haversian channels [82].

In addition to the above-mentioned advantages, sheep are very docile, cooperative, easy to handle and the cost of buying and its maintenance is not very high, thus allowing large-scale studies [327]. However, care should be taken with model planning, considering the differences that may exist in the bone structure, bone load and amount of underlying soft tissue, since these factors may influence the healing process [325]. More than that, our researchers' group has been working with this kind of model for more than a decade.

i) Microstructure of the bony tissue of the ovine model

While macroscopically sheep bones may have similarities to human bones, histologically the sheep's bone structure is quite different (**Figure 25 a-c**). Sheep have a predominantly primary bone structure compared to the largely secondary bone of humans [325]. In addition, Haversian remodelling (osteonal) in sheep (**Figure 25 d**) becomes more prevalent with age [325].

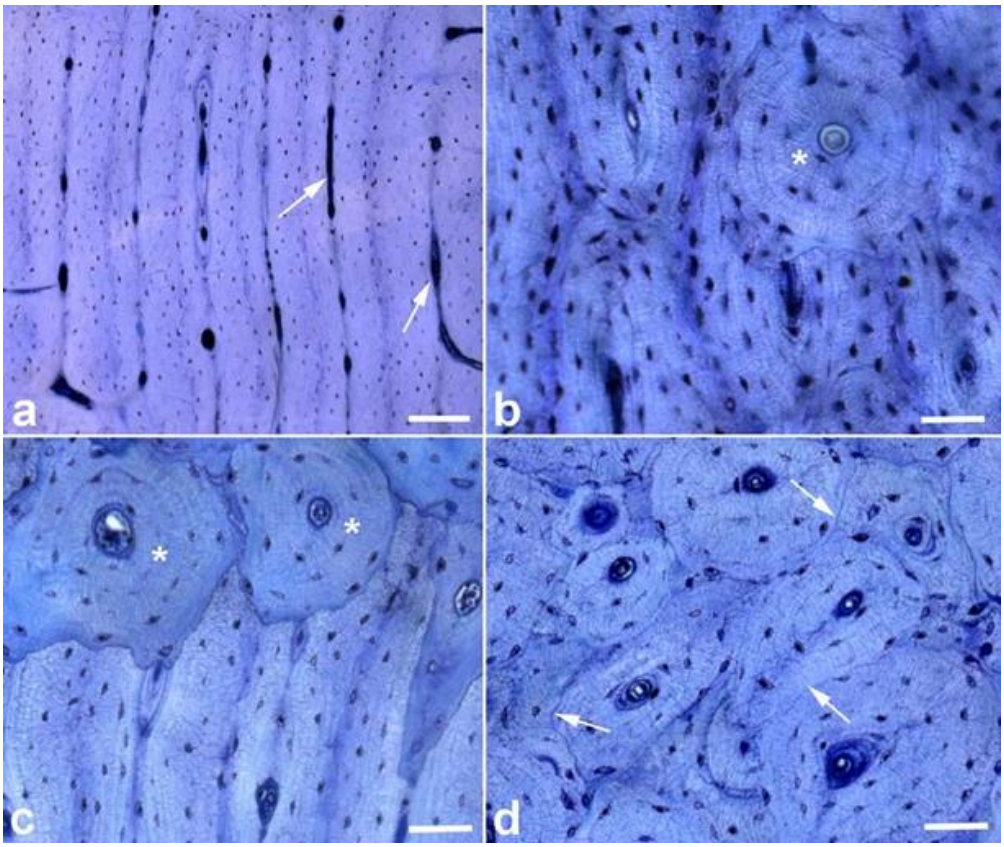


Figure 25 – Sheep, tibia: Dry and polished sections of MMA–embedded sheep tibia stained with toluidine blue. A) Plexiform or, as it is also called, laminar appearance of cortical bone with vessels arranged longitudinally (arrows) between bony lamellae. Scale = 200 μm. B) Remodelling of an area with originally plexiform bone that has been replaced by secondary osteons (*). Scale = 50 μm. C) Remodelling of the plexiform bone in the immediate vicinity of an implant. During healing and subsequent remodelling, the new bone is deposited as secondary osteons, seen at the top of the image (*). Scale = 50 μm. D) Cross section of the secondary bone with numerous osteons that can be clearly distinguished. It has lines separating neighbouring osteons (arrows). Scale = 50 μm [325].

ii) Bone composition

There are differences in the bone density between the human and the ovine, so the sheep bone shows a significantly higher density and therefore a higher resistance [325].

iii) Bone remodelling

Although differences in bone structure are recognized and sheep have a greater amount of bone growth than humans, it is proposed that this is due to the increased amount of spongy bone in the distal femur of the sheep compared to humans [325].

Studies have reported that measures of bone volume, osteoid volume, and mineral apposition rate of 9 to 10year old sheep are comparable to those of men and women in their sixties and seventies, suggesting that aged sheep may be considered to create models suitable for osteopenia and human osteoporosis [328].

Histomorphometric analysis

In order to evaluate the behaviour of the biomaterials in the bone regeneration verified in the animal models, histomorphometric analyses must be performed, in order to determine osteointegration, osteoconduction and degree of absorption of the biomaterial, which is a quantitative and rigours method developed by Atayde *et al.* [82]. It is necessary to calculate the area of the defect, the area of the different fractions (bone, connective tissue, void space and biomaterial) and contact areas between material and newly formed bone [21].

Several methods can be used to perform these histomorphometric tests such as: delimiting the boundary of the desired fraction, using counting grids and calculating fractional areas, using the segmentation of images (Thresholding). Image segmentation consists of separating the image by grouping pixels that have the same characteristic [21].

Atayde *et al.* [82] used the Image J® software (https://imagej.nih.gov/ij/) to proceed with image Threshold. Briefly, grey scale images are converted to 8-bit and run through the Threshold tool (Image/Adjust/Threshold). Minimum and maximum threshold values are defined to separate and measure fractions areas of the lacunae, new bone and biomaterial with the Measure tool on Image J (Analyse/Measure). At last, percentage (%) of Lacunae area [(defect Lacunae area /defect total area*100], new bone [(defect new bone area /defect total area*100], and biomaterial [(defect biomaterial area /defect total area*100]. In this study, it was verified that it is possible to select and isolate the tissue fraction and the area calculation, allowing to follow the progress of bone regeneration [21, 82].

Histological analyses

In addition to the histomorphometric analysis, a histological analysis should also be performed, allowing evaluation of bone quality and interaction between

biomaterials and bones. In these analyses, phases of bone healing may be observed, such as the initial formation of bone and trabecular bone, followed by the modelling and remodelling process [21].

The histological studies carried out in animal models, allow qualitative analysis of the type of interface between the biomaterial and the bone tissue. In the histological analysis of the samples containing the materials implanted in the tissues four different situations can be found: if the material is toxic the surrounding tissues undergo a process of necrosis; if the material is non-toxic and biodegradable, it may be replaced by the surrounding tissue or by a fibrous capsule; if the material is not toxic and is biologically inactive, a fibrous capsule is formed which surrounds it; and if the material is non-toxic and biologically active, it may form a bond at the interface with the tissue where it was implanted [72].

Several parameters can be used to quantify and qualify the behaviour of biomaterials: the quantification of the percentage of bone that interacts directly with the surface of the implants, the determination of the type and quantity of immunological cells that concentrate in the surrounding tissues of the biomaterial and the understanding of the phenomena and mechanisms that result from the implantation of biomaterials [72].

Histological studies based on optical microscopy, fluorescence, scanning electron and transmission, are often used as qualitative methods in the study of the behaviour of bone regeneration and the interface between the implant and the bone tissue. The histological analysis by optical microscopy is a rapid technique that allows to show the type of implant adhesion to the surrounding tissues because its resolution capacity is enough to characterize the interface and the intervention of soft tissue cells. However, it does not allow distinguishing physical contact from chemical adhesion at different stages of the integration process. On the other hand, optical microscopy only allows an overview of the tissues that form around the implants, and the characterization and description of the nature of the response of the bone tissue to implant placement should be studied in high resolution [21, 187].

Materials and methods

The *US Food and Drug Administration* (FDA) requires pre-clinical trials to be performed on a large animal model, considering medical devices like bone substitutes. The *in vivo* tests were performed at the Centro Clínico de Investigação Veterinária de Vairão (CCIVV) of University of Porto (ICBAS-UP) (**Figure 26**, **Figure 27** and **Figure 28**), authorized by the National Authority, Direção-Geral de Alimentação e Veterinária (DGAV) to animal experimentation. The *in vivo* experiments were approved by the Committee Responsible for Animal Welfare (ORBEA) of ICBAS as well as by DGAV (projects nº 178/2017 and nº 192/2017). All procedures were carried out with the approval of the veterinary authorities of Portugal (DGAV) in accordance with Directive 86/609 / EEC of the Council of the European Communities. All the animals were identified with an earring and vaccinated, according to DGAV instructions.

The interaction of Bonelike® in bone tissue was evaluated in 12 ovines (*Ovis aries*), divided in 6 males and 6 females, Merino breed, weighing approximately 50 kg and aged between 7 and 8 years (**Figure 26-29**).



Figure 26 – A) Centro Clínico de Investigação Veterinária de Vairão; B) Surgical preparation room.

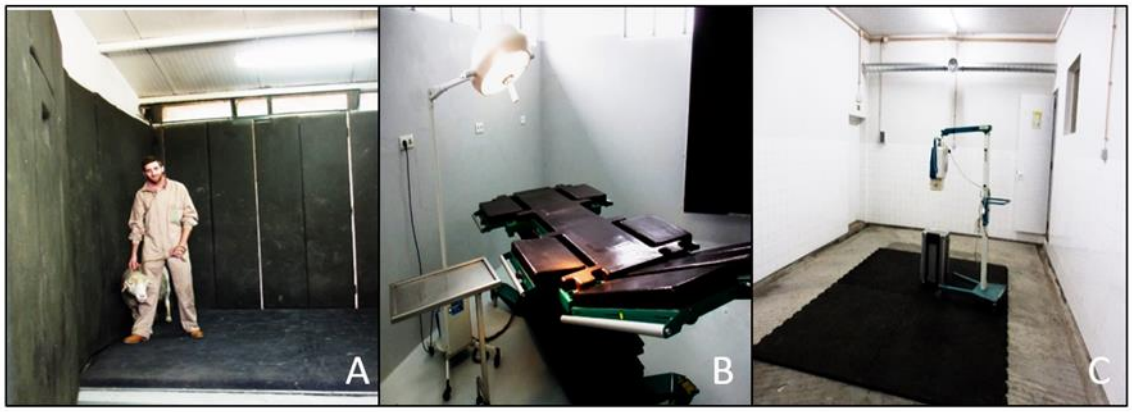


Figure 27 – A) Anaesthetic induction room; B) Operating room; C) Radiography room.



Figure 28 – A) Ovine parks; B) Recovery metabolic cages.



Figure 29 – A) Sheep Merino breed; B) DGAV earring identification.

Anaesthesia procedure

In order to use a multimodal anaesthesia, an injection of pre-medication was administered intramuscularly in the animal's lumbar muscles, containing a mixture of an α-2 agonist called xylazine (Rompun®) dose of 0.1 mg / kg together with an opioid agonist-antagonist, butorphanol (Toburgesic®) dose of 0.05 mg / kg.

This initial sedation allowed the shaving in the regions of both hind limbs (**Figure 30**). Thus, first washing and initial asepsis of the animal was performed with iodopovidone 10% solution foam (Betadine®). After the placement of the 20 Gauss intravenous catheter (B Braun®) in the left cephalic vein of the sheep for the intraoperative fluid administration (NaCl 0.9% B Braun®), infused at a maintenance rate, it was possible to perform an anaesthetic induction by intravenous administration of tiletamine-zolazepam (5.5 mg / kg, Zoletil50®). Animals walked into the surgical preparation room and a second washing was performed with iodopovidone 10% solution foam (Betadine®).



Figure 30 – A) Superficial shear at the animal park; B) Deep shear performed at the animal park; C) In route to surgical preparation room; D) Hind limbs and lumbosacral region scrub.

The sheep already in sedation, was placed on the operating table in the sphinx position for a loco-regional anaesthesia, using a spinal catheter. Anestesin® 2% (20 mg / ml lidocaine hydrochloride) was used as the anaesthetic for the epidural anaesthesia (**Figure 30 A**). At that time the animal was placed in lateral decubitus and a third and last aseptic cleaning of the operative area was performed. An endotracheal tube was placed in the mouth to allow better ventilation trough the upper airways (**Figure 31 B**).

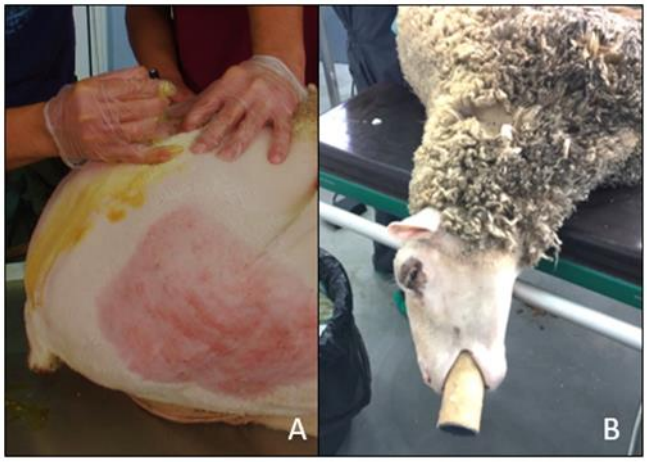


Figure 31 – A) Epidural technique in sphinx position; B) Application of an endotracheal tube.

The monitoring of the cardiorespiratory parameters throughout the entire surgical intervention was performed and recorded on the appropriated anaesthetic sheet (see attachments) [23, 54]. In some surgeries, intravenous puncture of tiletamine-zolazepam (5.5 mg / kg, Zoletil50®) was administered intravenously (**Figure 32 A**). A bandage covering the tail avoid intra-operative contaminations was performed in all surgeries (**Figure 32 B**).

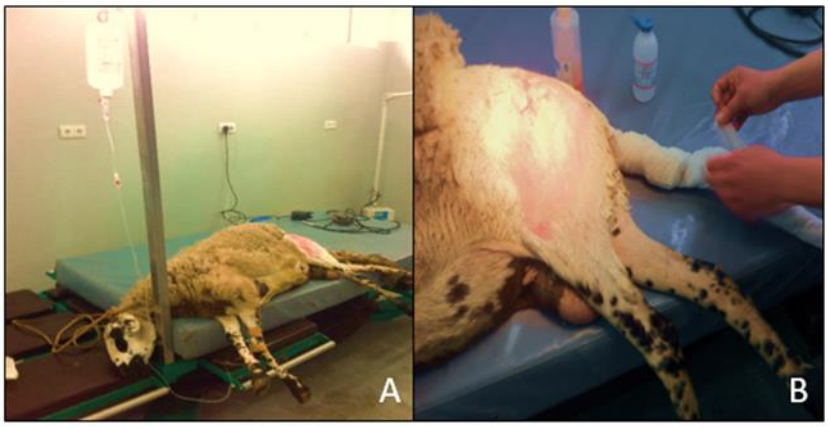


Figure 32 – A) Fluid therapy and positing lateral recumbence; B) Tail bandaging for protection.

The operative area is covered by a sterilized compress until the beginning of the surgery (**Figure 33 A**). The surgery begins with an incision on the skin (**Figure 33 B**). At the end of all surgical procedures, atipemazol (Antisedan Zoetis®) dose of 0.05 mg / kg was administrated intramuscularly on their lumbar muscles. Atipemazol is a synthetic α-2 adrenergic receptor antagonist, indicated for the reversal of the sedative effects of agonist (xylazine) administered before for anaesthesia induction.

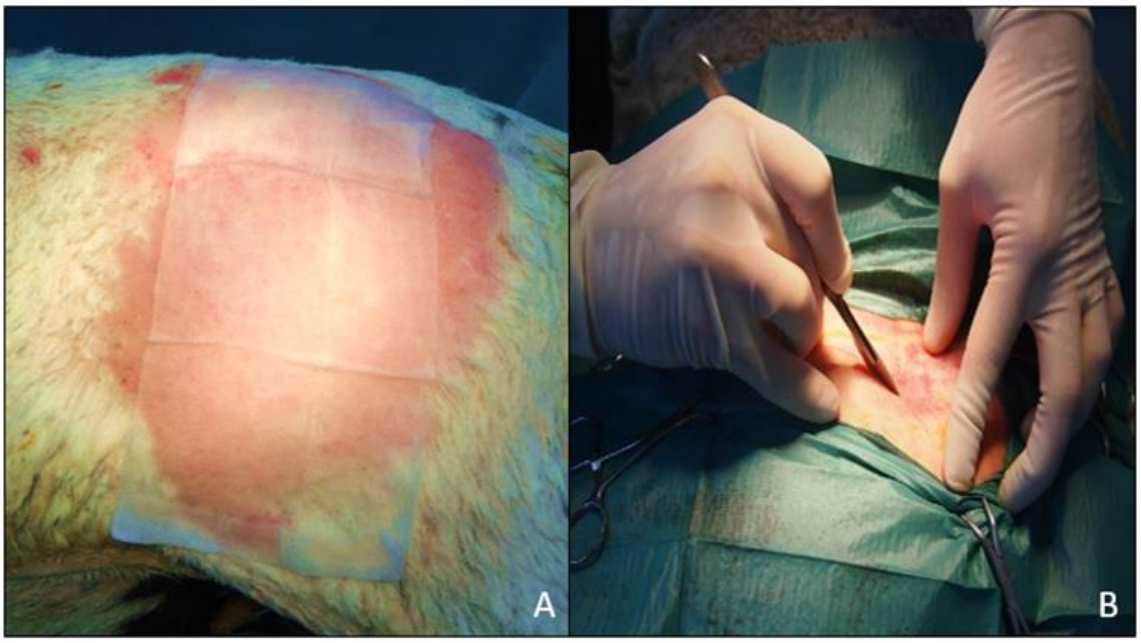


Figure 33 –A) Aseptic operating site; B) Skin incision.

Surgical Procedure: standardized non-critical defects performance

A surgical incision was made using a blade (Braun® carbon steel) size 24, approximately 15 cm in length, from proximal to distal on the skin, from the greater trochanter to lateral epicondyle of the femur, Followed by a skin incision over the lateral aspect of the femur (**Figure 34 A**). Incision of the exposed *fascia lata* (**Figure 34 B and C**) allows delineation of the *vastus lateralis* muscle cranially and *biceps femoris* muscle caudally. These are separated to reveal the lateral diaphysis of femur. A limited lateral approach to the diaphysis, of sufficient size to adequately visualize de lateral aspect of femur, should then be performed (**Figure 35 A**).

The adductor muscle attachments to the caudal femur are left undisturbed since these provide an important extra-osseous blood supply to the bone. Thus, through a blunt dissection and without risk of damaging adjacent soft tissues, it was

possible to access and expose the femur using retractors. Five holes were drilled with a 4.7 mm diameter drill, creating non-critical bone defects with about 5 mm diameter (**Figure 35 B**). Retractors allow visualizing the femoral diaphysis and its orifices (**Figure 35 C**).

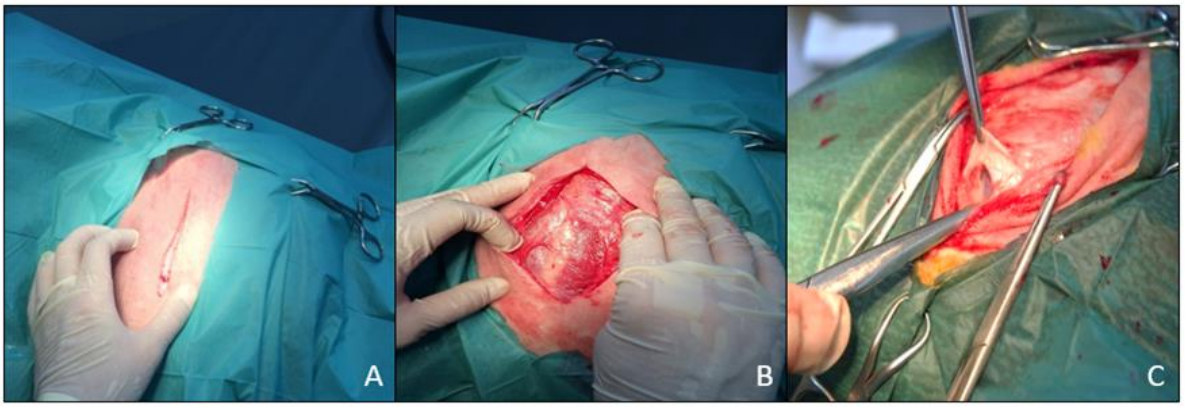


Figure 34 – A) Incision over the lateral side of femur approximately; B) Identification of *fascia lata*; C) Incision over *fascia lata*.

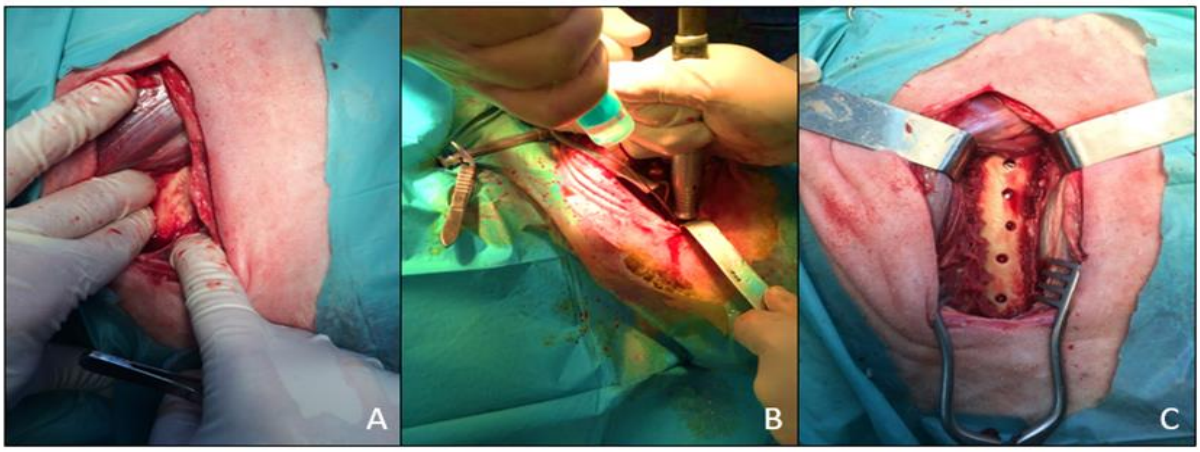


Figure 35 – A) Muscles retraction and femur exposure; B) Drilling and flushing of 5 bone defects on exposed femur; C) Muscles retraction exposing the bone defects.

These dimensions were chosen because it represents a non-critical size defect. Each bone defect was performed with 1 cm distance between each hole in order to reduce the risk of fragility of this mono-cortical model and thus prevent fractures. During the drilling of these defects, the drill bit is flushed by sterile saline 0.9% (Braun® B), thereby preventing bone damage and bone necrosis due to high temperatures reached during that procedure, as well as removing residual bone.

The first defect created (from dorsal to ventral direction of the femur) was not filled to be used as void control (Control). The second defect was filled just with the

biomaterial (Bonelike®) and the remaining three defects were filled with Bonelike® *plus* hDPSCs (prepared at the Laboratory of Veterinary Cellular Therapies of ICBAS-UP) (**Figure 36**).

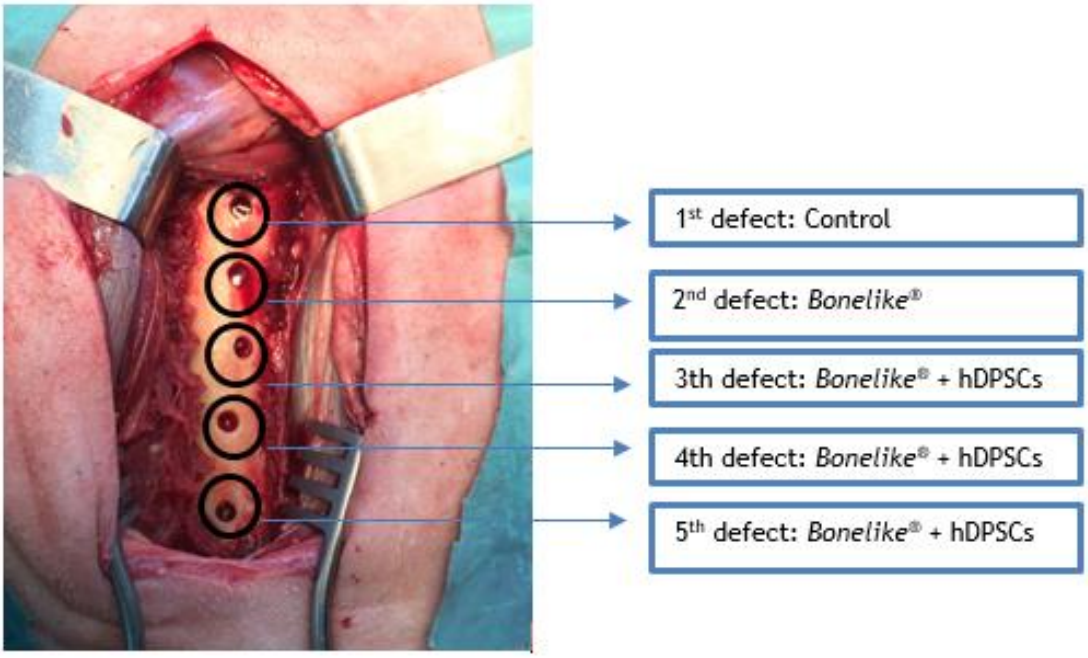


Figure 36 - Ovine, femur: Lateral exposure of a femoral diaphysis with 5 non-critical defects.

Application of biomaterial and hDPSCs

Prior to filling the four distal holes with Bonelike® and Bonelike® plus hDPSCs, Bonelike® (250-500 μm) (**Figure 37**) was mixed with Tisseel® (Baxter®) (**Figure 38**). Tisseel® is a fibrin sealant indicated as an adjunct to standard surgical techniques (such as suture and ligature) to prevent leakage or use as an adjunct to haemostasis in patients undergoing surgery when control of bleeding by conventional surgical techniques (such as suture, ligature, and cautery) is ineffective or impractical. Is also called a natural glue of human origin for tissues, composed of two components, namely the sealant protein solution (fibrinogen) and the thrombin solution. These are two of the blood proteins that are important for the coagulation of the same [329].



Figure 37 – Sterilized Bonelike®.

The fibrin clot formed by Tisseel® is very similar to the clot formed in normal blood coagulation. It is degraded by the same pathway as the endogenous clot (of the organism) and leaves no residue or by-product. Accordingly, a defect filling material, prepared primarily of collagen, which has improved mechanical stability with adequately density and sufficiently conformable for surgical utility is necessary for implantation of the cell-based therapies, that will work as a cell vehicle [17].



Figure 38 – Syringes of Tisseel®.

In this case, the important function of Tisseel® was fixing the Bonelike® and the hDSPCs together inside the mono-cortical defect created before. After the Bonelike® and Tisseel® blend (**Figure 39 A and B**), the mixture was applied to defects number

#3, #4 and #5 (**Figure 40 A and B**) and $10x10^4$ hDPSCs were added (**Figure 41 A and B**).

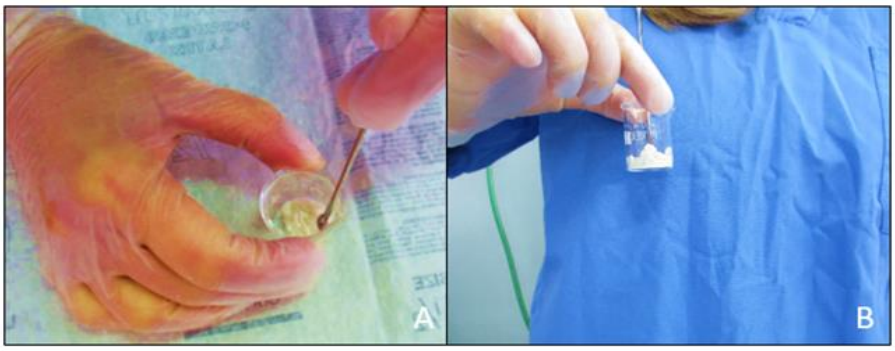


Figure 39 –A) Bonelike® blend with Tisseel®; B) Prepared bone graft.

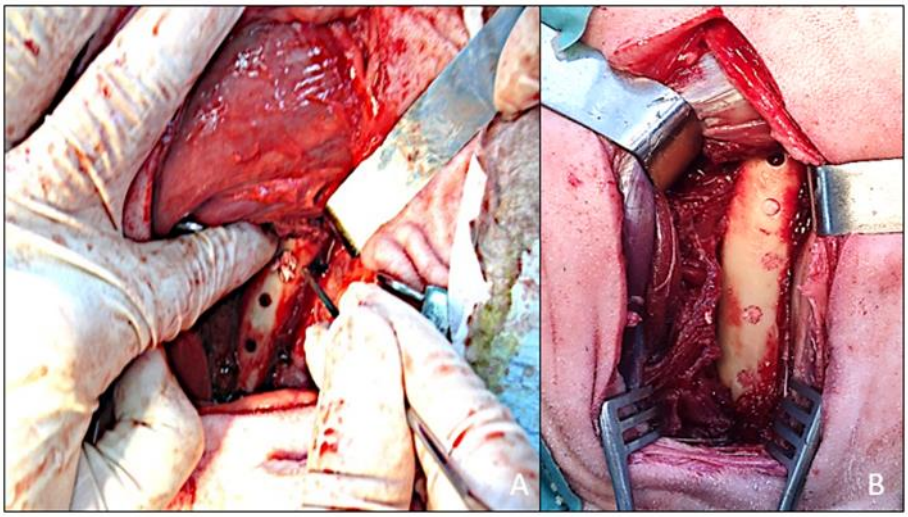


Figure 40 – A) Filling the bone defect; B) Filled bone defect.

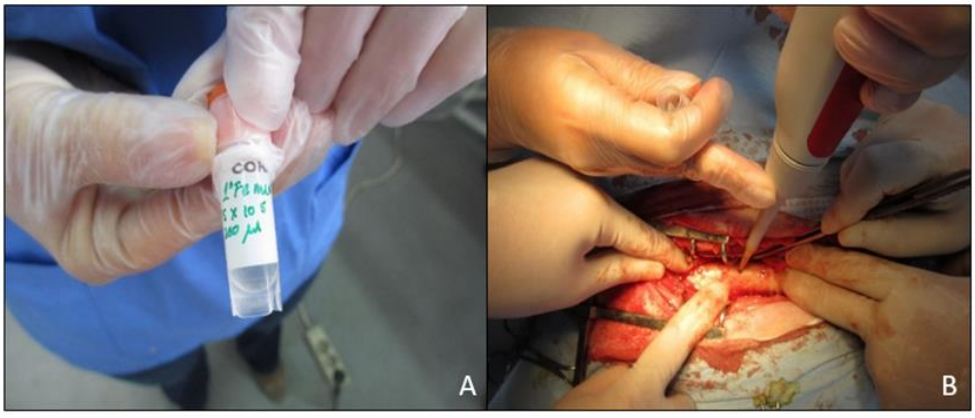


Figure 41 – A) Sample of hDPSCs; B) Application of hDPSCs inside bone defects.

The *fascia lata* was closed with an absorbable suture material (Monosyn® 1/0 with triangular needle Braun®) (**Figure 42 A and B***)* in a simple continuous pattern

suture, thereafter closure with an absorbable suture material (Monosyn® 2/0 triangular needle Braun®) in a simple continuous pattern suture for the adjacent subcutaneous soft tissue, and finally a continuous pattern suture in the skin *(***Figure 42 C and D***).* At the end, the suture was sprayed with an antibiotic Terramicina spray Pfizer® to prevent local bacterial infections. This surgical procedure was repeated on both hind limbs of each experimental animal.

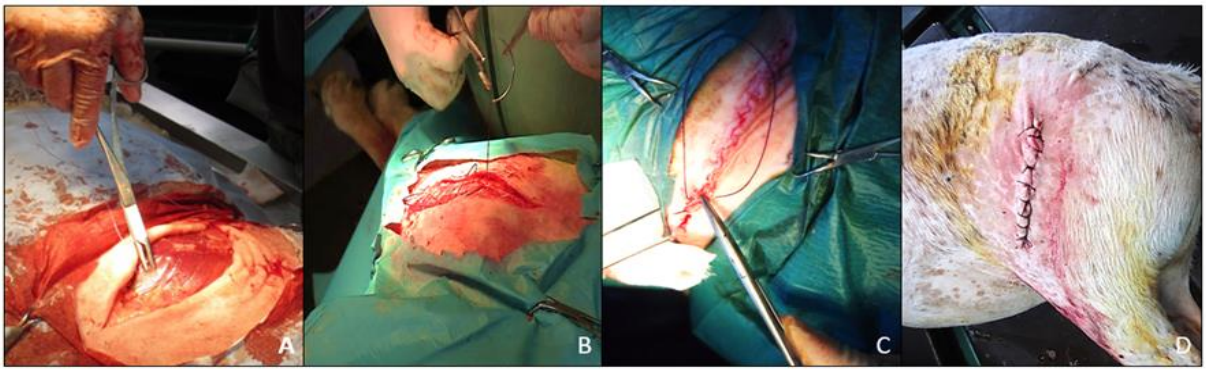


Figure 42 – A) Closing *fascia* lata; B) Closing subcutaneous tissue; C) Approximation skin suture; D) Skin suture.

Post-surgical procedure

In order to prevent wound contamination, on all surgical wound, were applicate Terra-Cortril®, that is an antibiotic spray with hydrocortisone, a steroid anti-inflammatory (Terramicina spray Pfizer®) (**Figure 43 A**). The animals remained in metabolic cages for the first 24 hours and then were transferred to parks with 2 or 3 ewes (**Figure 43 B and C**). Hoof care is an important aspect of sheep management and was considered before place animals in the park and continuously supervised during the healing period after the surgeries (**Figure 44 A and B**).



Figure 43 – A) Application of Terra-Cortril® on surgical wound – An antibiotic spray (Terramicina spray Pfizer®) and steroid anti-inflammatory (hydrocortisone); B) Placement of the sheep in the recovery cage; C) Placement of the sheep in the park.

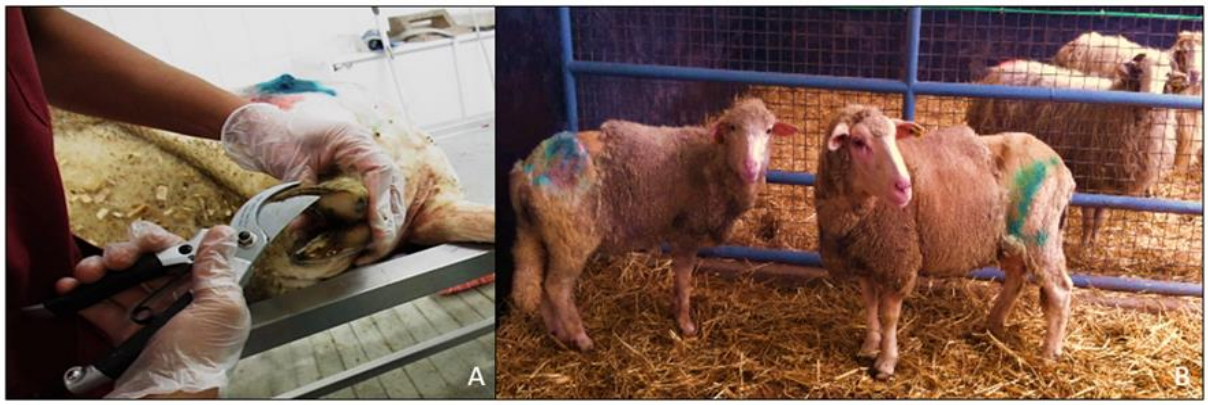


Figure 44 – A) Hoof trimming to improve locomotion; B) Groups of sheep in the park months after surgical procedure.

An intramuscular preventive antibiotic injection Pendistrep® (penicillin and streptomycin) (**Figure 45**) and non-steroidal anti-inflammatory drug injection (Rimadyl® – Carprofeno, doses 0.7mg / kg) were given postoperatively for each surgery in order to prevent infections and confer analgesia, respectively. Both injections were repeated, intramuscularly on the neck's lateral muscles, every day for 5 days. Important data was registered on the hospitalization sheet form (see attachments).



Figure 45 - Intramuscular administration of antibiotic and non-steroid anti-inflammatory drug.

The euthanasia of the animals was done in a humane and respectable way at a calm location isolated from the rest of the animals with an intravenous administration of sodium pentobarbital 20% (Eutasil®, Ceva). The sacrifice of those animals was made at different times of implantation / healing period of 30, 60 and 120 days. After the animals were euthanized, the femurs were removed and adjacent soft tissues were cleaned from the femurs which were immediately preserved in 10% formaldehyde for subsequent cutting of the bone segments for histological analysis and histomorfometric analysis.

Radiographs

There were two moments of the healing period in which the radiographs were performed. The first one was immediately after the surgery to confirm that the distance between defects and alignment of those created defects was correct and the defects were correctly filled or left empty (**Figure 46**).

The second moment using X-ray image, was immediately *post mortem.* After the femurs removal from the body, radiographs of lateral and medial incidence were performed on the femur immediately after euthanasia, at 30, 60 or 120 days of healing period (**Figure 47**).

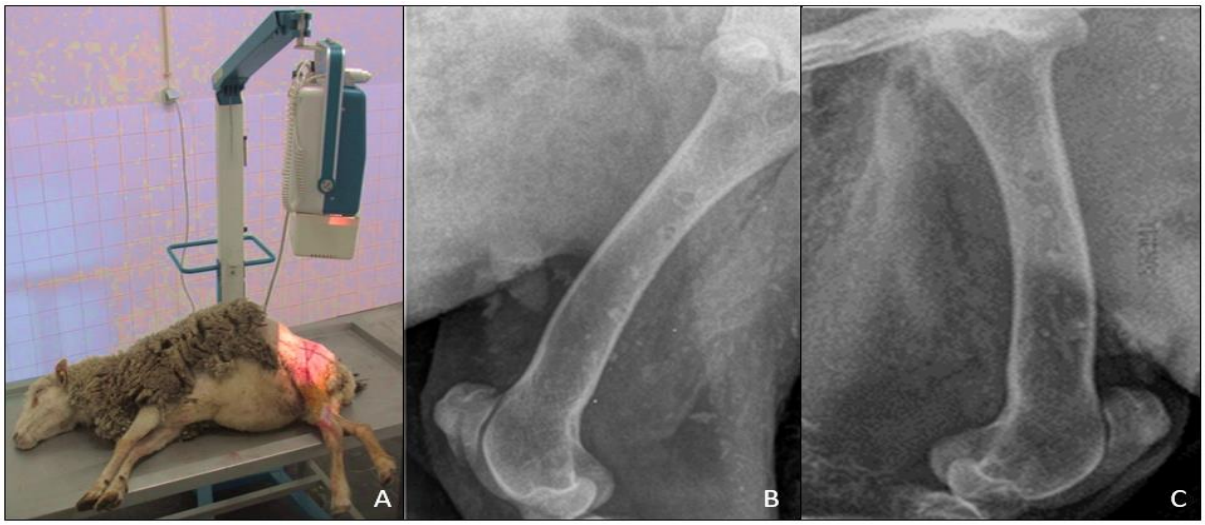


Figure 46 – A) Ovine, femur: Latero-lateral radiograph of two femurs after surgery; B) and C) – Ovine, femur: Radiographs after surgery.

The X-ray used was SCANCO Medical AG, model A6583-01 with a conical beam, from an X-ray tube with a focal point of 7 μm. The realization of these X-rays allowed to visualize the difference of mineralization in the different holes without being necessary the use of tools or computer software that allow to draw conclusions beyond the macroscopically presented. Another objective of post-mortem radiographs was to allow the identification of the holes and, therefore, a safety cut of the bone segments to be sent for pathological analysis.

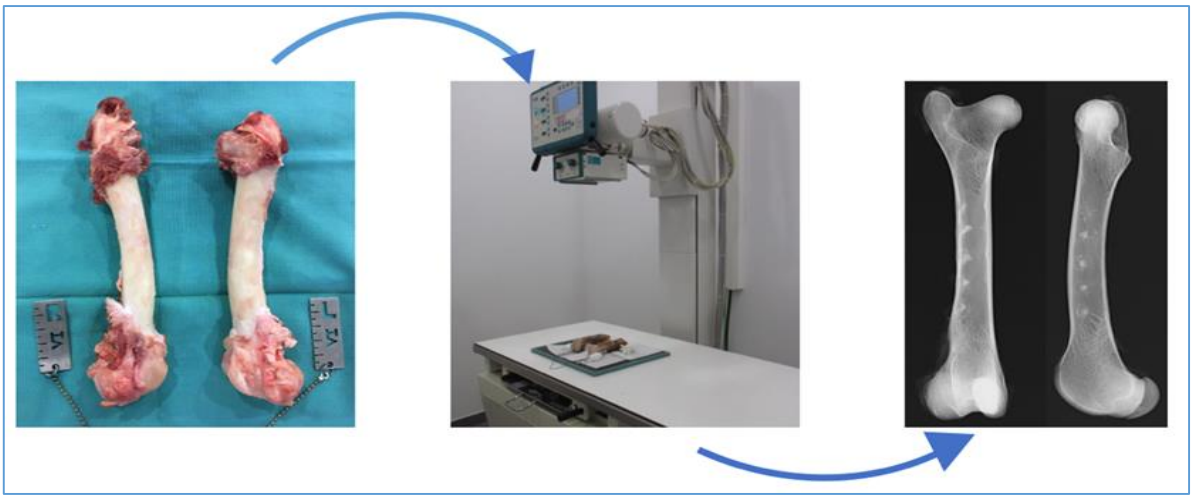


Figure 47 – Ovine, femur: Schematic of lateral-medial-to-femoral (post-mortem).

Sample preparation for further staining and SEM analysis

Then, osteotomies of the femoral neck and head of the both bones were performed after cleaning bones in order to obtain the femoral diaphysis. The bone

marrow was gently removed to obtain the internal cavities where the bone defects are. After locating the bone defects, two transverse lines were designed to serve as cutting guides (**Figure 48 A**). Segments with defects between the drawn lines were cut with an oscillating cutting saw (Bosch Professional® 300W) perpendicular to the long axis of the femur and flushing with a sterile saline solution to minimize thermal damage (**Figure 48 B and C**). After these procedures, the samples were immersed in neutral formaldehyde (10%) and maintained for seven days refrigerated temperatures.

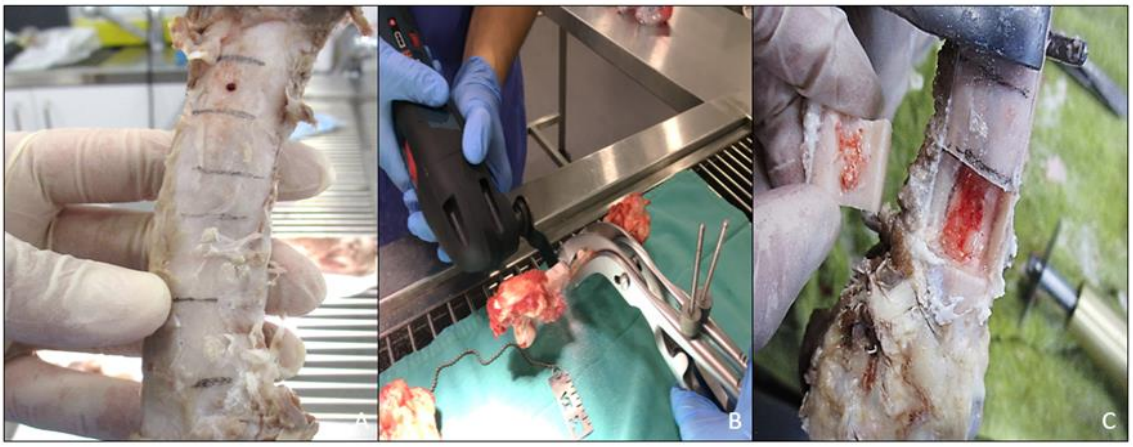


Figure 48 – A) Guide lines to allow cutting the edges; B) Oscillating saw; C) Square of bone defect.

The samples were organized (**Figure 49 A and B**) to be immersed in increasing percentages of alcohol (alcohol 70%, 80%, 90%, 96% and 100%, one week in each holder concentration) for dehydration.

Thereafter, they were incorporated into a methylmethacrylate resin for one week for impregnation and one more week for inclusion. Finally, the sample is washed and ready to be cut by the microtome. Using a microtome with a diamond blade, thin sections with 150 μm of specimen were made perpendicular to the axis of the femur.

Then the samples were delivered at Faculdade de Medicina Dentária da Universidade do Porto (FMDUP), Anatomy Laboratory (**Figure 49 C**). Histomorphometric analysis and histological process could procced after this preparation.

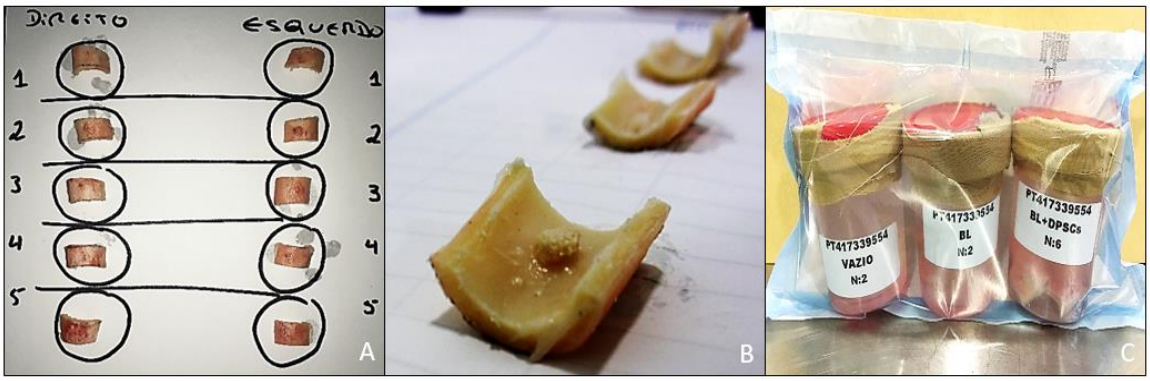


Figure 49 – A) Organization of pieces of bone; B) Sample of bone cutter; C) Packaged samples for shipping Samples to FMDUP.

Histological analysis

Samples were placed in a neutral formaldehyde fixative solution (10%) for 7 days, dehydrated in an increased percentage of alcohol solutions, and embedded in a methylmethacrylate resin. Thin sections with 150 lm were cut perpendicularly to the femur axis with a diamond blade microtome and hand-ground to approximately 70–80 lm.

Some sections were stained with Solochrome Cyanine R for histological examination on a Nikon microscope (Eclipse E600, Nikon, Tokyo, Japan), equipped with a calibrated digital camera (Nikon DS-5M-L1 Digital Sight Camera System, Nikon). This dye differentiates osteoid from neo-formed bone and old bone [90]. In the following table it is described the colours that can be found in the analysis of the images with the dye Solochrome Cyanine R (**Table 4**).

Table 4 – Correspondence of the expected colours when blushing with the histochemistry of Solochrome Cyanine R (Adapted from [86]).

Tone / Expected Results	Corresponding fabrics / structures
Light blue	Mineralized bone
Dark blue	Calcification front
Light Orange Red	Osteoid
Blue	Nuclei

The histological images obtained from the stain with Solochrome Cyanine R were used to evaluate the cellular reaction and the amount of neo-formed bone, allowing the characterization of the bone formation events and the remodelling

process during the healing process. Based on histological analysis, the biomaterial can be classified as: toxic, bioinert, bioactive, and bioreabsorbable.

Histomorphometric analysis – Thresholding

On the other hand, the uncoloured sections were used to perform histomorphometric analysis by SEM analysis. Primarily samples were recovered with gold to be prepared for SEM (**Figure 50 A and B**).

The SEM / EDS test was performed using a high-resolution (Schottky®) Scanning Electron Microscope with X-ray microanalysis and analysis of Rectangular Electron Diffraction Patterns: Quanta 400 ESEG / EDAX Genesis® X4M FEG, operating in high vacuum mode with an acceleration voltage of 15kV SEM. reabsorbed and gradually replaced by new bone (CEMUP) (**Figure 51 A and B**).

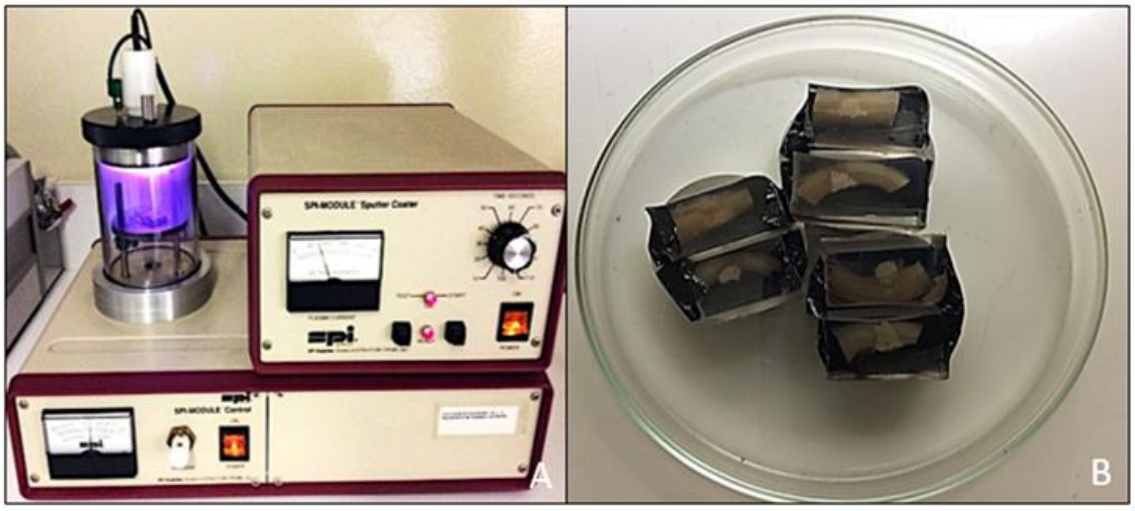


Figure 50 – A) Recovering sample with gold; B) Samples prepared for SEM.

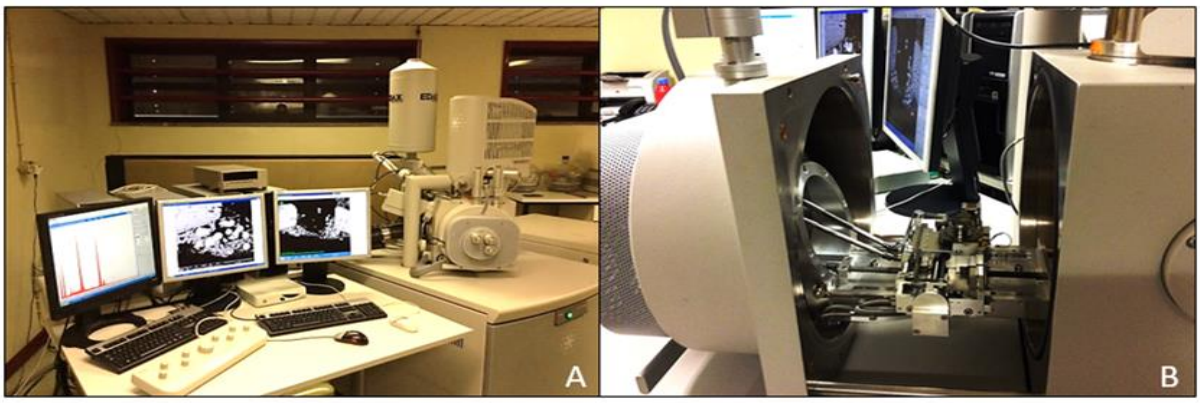


Figure 51– A) Scanning Electron Microscope (CEMUP); B) Sample going inside the SEM.

Thus, to complement the histological analysis, a histomorphometric analysis was performed, allowing the evaluation of the bone quality and the interaction between the biomaterial and the bone.

To evaluate the behaviour of the biomaterials in these animal models, we must perform the histomorphometric analysis. Several methods can be used to perform these histomorphometric analysis, some of them laborious, such as delimiting the contour of the desired fraction or the use of counting waists. Another less labour-intensive alternative to calculating fraction areas is using image segmentation (Thresholding). It should be emphasised that histomorphometry is considered the "gold standard" method for evaluation of bone structure in osteointegration research [77].

Each SEM image was divided in Photoshop® into three fractions: the periosteal callus, the defect area, which corresponds to the cortical callus and the endosteal callus (**Figure 52**). Then, an independent analysis was performed on each image.

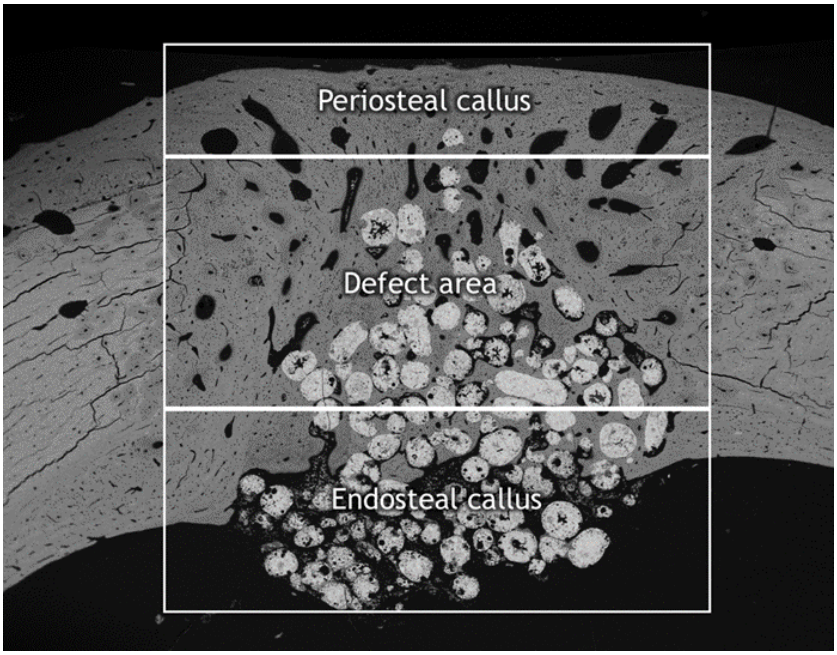


Figure 52 – Ovine, femur: SEM image divided into three different fractions: periosteal callus, defect area and endosteal callus (total magnification: 30x).

Atayde *et al.* [91] developed a rapid, objective and reproducible method for the histomorphometric analysis of regenerated bone tissue, consisting of the semiautomatic segmentation (Thresholding method) of histological images [73]. The segmentation of the image consists of the separation of the image, grouping pixels

that have the same attribute. In order to proceed with image segmentation, Image J® software was used.

With this method, it is possible to separate and measure the area of fracture of the gaps, neo-formed bone, biomaterial and unfilled areas. The separation of the different areas is done by dividing the histogram as we can see in **Figure 53.**

Through these parameters, it is possible to monitor and evaluate bone growth by comparing the results obtained in the biomaterial, the biomaterial with the hDPSCs and in the void control. The degree of resorption of the biomaterial can be quantified by this method, comparing the variation of the area occupied by the biomaterial / biomaterial *plus* hDPSCs throughout the times of implantation. It is also possible to compare the bone growth considering the implantation time, in the different fractions of the image (the defect area, the periosteal callus and the endosteal *callus*).

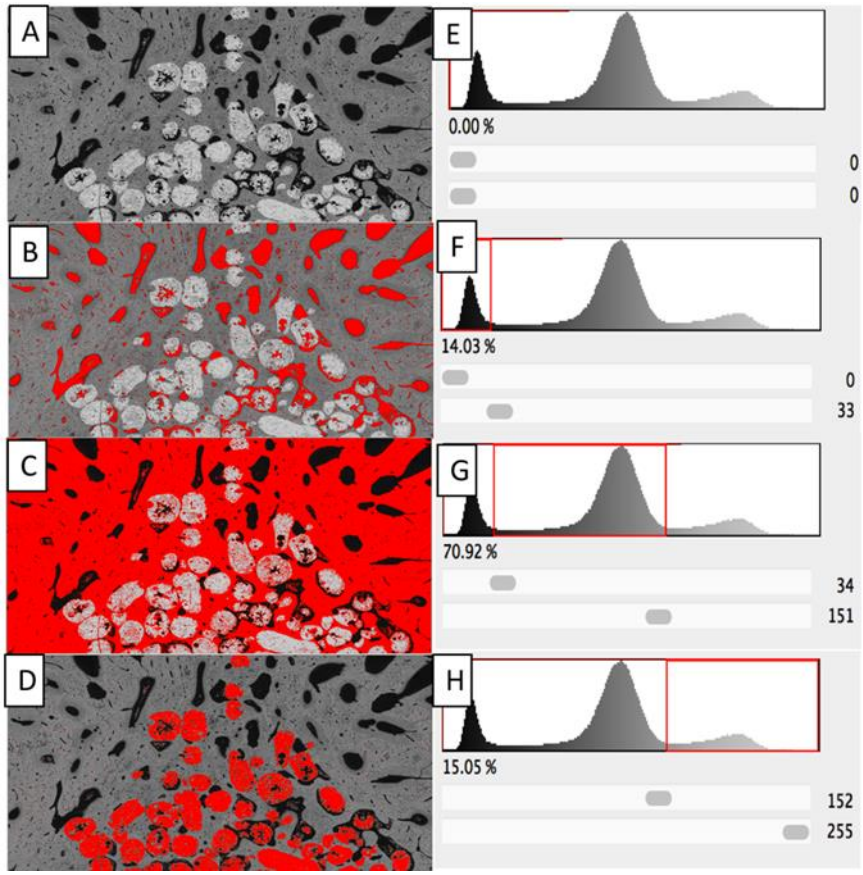


Figure 53 – Ovine, femur: Threshold analysis of a SEM image of bone tissue where the Bonelike® (A – D) was implanted. The image segmentation was done using the histogram (E – H). A – Image to analyze; B – Selection of gaps; C – Selection of bone; D – Biomaterial selection.

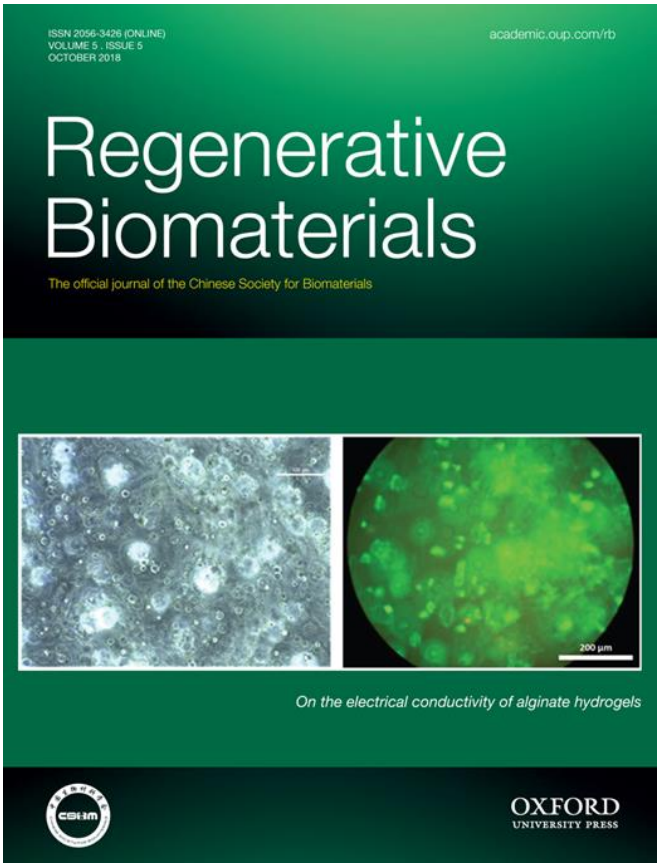
Statistical analysis

Statistical analysis was performed using GraphPad Prism® version 6.00 for Mac OS X, GraphPad Software, La Jolla California, USA. The results were presented as mean ± standard deviation (SE). The analysis was performed by the unidirectional ANOVA test followed by the Tukey multiple comparisons test. The differences were considered statistically significant at $P \leq 0.05$.

The meaning of the results is shown in comparison with the other groups and represented by the symbol (*). The meanings of the results are also indicated according to the values of P (probability of significance) with one, two, three or four of the symbols (*) corresponding to 0.01 <P0.05, 0.001 <P0, 01, 0.0001 <P≤0.001 and P≤0.0001, respectively.

Chapter 7 – Dental Pulp Stem Cells and Bonelike® for Bone Regeneration in Ovine Model

The osteogenic potential of DPSCs associated to a modified HA bone substitute (Bonelike®) was evaluated *in vivo* in a monocortical ovine model that consists in 5 holes (5 mm Ø), in the femoral diaphysis. I performed dozens of surgeries and the results were obtained, analyzed and studied over three years of work.



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Research Article

OXFORD

Dental pulp stem cells and Bonelike® for bone regeneration in ovine model

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Abstract

Development of synthetic bone substitutes has arisen as a major research interest in the need to find an alternative to autologous bone grafts. Using an ovine model, the present pre-clinical study presents a synthetic bone graft (Bonelike®) in combination with a cellular system as an alternative for the regeneration of non-critical defects. The association of biomaterials and cell-based therapies is a promising strategy for bone tissue engineering. Mesenchymal stem cells (MSCs) from human dental pulp have demonstrated both *in vitro* and *in vivo* to interact with diverse biomaterial systems and promote mineral deposition, aiming at the reconstruction of osseous defects. Moreover, these cells can be found and isolated from many species. Non-critical bone defects were treated with Bonelike® with or without MSCs obtained from the human dental pulp. Results showed that Bonelike® and MSCs treated defects showed improved bone regeneration compared with the defects treated with Bonelike® alone. Also, it was observed that the biomaterial matrix was reabsorbed and gradually replaced by new bone during the healing process. We therefore propose this combination as an efficient binomial strategy that promotes bone growth and vascularization in non-critical bone defects.

Keywords: biomaterial; Bonelike®; bone regeneration, dental pulp; human bone; hydroxyapatite; mesenchymal stem cells; ovine model; tissue regeneration; tricalcium phosphate

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Introduction

In recent years, the average life expectancy of the European population has increased [1] and so has the number of degenerative diseases, osteogenic disorders and bone fractures [2]. More than two million bone grafts were performed throughout the world, making bone grafts the second most performed tissue transplant after blood, in the last decades [3, 4]. According to their origin, bone grafts can be classified as autografts (originating from the individual), allografts (from another individual) and xenografts (from another species) [5]. These different types of bone grafts may present some drawbacks such as limited quantity and availability, donor morbidity and disease transmission. Therefore, alternatives are necessary to overcome these limitations. Synthetic bone substitutes, mainly based on hydroxyapatite (HA) and tricalcium phosphate (TCP), are a good alternative and have been the target of much research and development in recent years. These substitutes replicate the biological properties of natural bone without the disadvantages stated above. Natural bone morphology and properties are usually used as a model in the development of the ideal bone replacement [6], especially with regards to osteoconductive, osteogenesis and osteoinductive properties [3, 7]. The main application of synthetic bone substitutes is to become an alternative in the tackling of critical and non-critical bone defects [8]. Santos and collaborators [9–11] developed a synthetic bone substitute that has been patented and it's commercially available as Bonelike® (Biosckin S.A., Portugal) [8–11]. Bonelike® is composed of modified calcium phosphate and controlled proportions of HA, TCP and ionic species, to mimic the chemical and structural composition of human bone [12]. Bonelike® presents enhanced bone adhesion, cellular proliferation, and differentiation properties, and is reabsorbed gradually in a controlled way, as in natural bone remodelling [9–11]. Atayde et al. [6] recently demonstrated several advantages of Bonelike®, including easy extrusion through a syringe, and improved osteointegration, osteoconduction and degradation due to the presence of larger pores and a spherical format, which can adapt to bone growth.

The use of osteoconductive biomaterials with mesenchymal stem cells (MSCs) allows the proliferative and differentiation capacities of the latter to work in synergy with scaffolding properties of biomaterials. Human MSCs (hMSCs) characteristics have been defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) [13] as being plastic-adherent when maintained in standard culture conditions, expressing cluster of differentiation (CD) 105, CD73 and CD90, and lacking expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and human leucocyte antigen (HLA)-DR surface molecules. The lack of expression of HLA-DR surface molecules allows their application in other species without the risk of rejection by the receptor and the need of immunosuppression. Human dental pulp stem cells (hDPSCs) are isolated from dental pulp tissue and are a good source MSCs due to their accessibility, availability throughout life, high proliferation and differentiation capabilities, especially towards the osteogenic lineage and promotion of mineral deposition both *in vitro* and *in vivo*. Furthermore, hDPSCs are capable of secreting bioactive factors (growth factors and cytokines) that modulate the activity of native cells and inflammatory populations [14, 15].

In the present study, hDPSCs were characterized and expanded *in vitro* and concurrently implanted *in vivo* with Bonelike®, in standardized non-critical bone defects, for pre-clinical trials using an ovine model. The ovine model was selected because of its phylogenetic proximity to humans in terms of musculoskeletal size and mechanical characteristics. The presence of *Havers* channels and the process of cortical remodelling of bone structure, *plus* the docile temperament of this species, are further advantages. Other important considerations are the low acquisition and maintenance costs of sheep, and the ethical and social approval of their use compared with other mammals [16]. The aim was to monitor and evaluate bone regeneration and remodelling of Bonelike® *plus* hDPSCs over time in non-critical defects, including the examination of biomaterial reabsorption, bone growth and structure of regenerated areas using histological processing and histomorphometric analysis.

Materials and methods

Bonelike® preparation

The Bonelike® were prepared as detailed in [6]. Briefly: HA and P_2O_5-CaO based glass were individually prepared and mixed. HA was prepared through a precipitation method consisting of the reaction between calcium hydroxide ($Ca(OH)_2$) and ortho-phosphoric acid ($H_3(PO_4)_2$). A P_2O_5–CaO based glass with the composition of $65P_2O_5$- 5CaO-10CaF_2-10Na_2O in mol% was prepared from reagent grade chemicals by using a platinum crucible heated at 1450°C. Bonelike® was obtained by adding 2.5 wt% of glass HA and mixed with a pore forming agent. The mixture was extruded and spheronized and the pellets sintered. Standard sieving techniques were used to obtain the 250–500 μm particle size ranges.

To characterize the Bonelike®, a scanning electron microscopy (SEM) was used. The SEM exam was performed using a High-resolution (Schottky) Environmental SEM with X-ray microanalysis and electron backscattered diffraction analysis: Quanta 400 FEG ESEM/EDAX Genesis X4M®.

hMSC preparation

Cell culture and maintenance

hDPSCs were obtained from AllCells, LLC (Cat. DP0037F, Lot No. DPSC090411-01). Cells were thawed and expanded *in vitro* using standard protocols previously reported [17–29]. hPDSCs were maintained in αMEM, with GlutaMAX™, without nucleosides (32561029, Gibco®) supplemented with 10% (v/v) fetal bovine serum (A31608-02, Gibco®), 100 IU/ml penicillin, 0.1 mg/ml streptomycin (15140122, Gibco®), 2.05 μg/ml amphotericin B (15290026, Gibco®) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid Buffer solution (15630122, Gibco®), kept at 37°C and in a 95% humidified atmosphere with 5% CO_2.

DPSC characterization

DPSC phenotype identity. Surface marker profiles of hDPSCs were assessed by flow cytometry. hDPSCs populations were cultured for 5 days, as described previously [30], harvested using Accutase™ Cell detachment solution (561527, BD Biosciences©), being counted and resuspended in Stain Buffer (554676, BD Biosciences©). hDPSCs in Passage 5 and 7 (P5 and P7) were incubated with anti-positive (CD90, CD105 and CD44) and anti-negative marker (CD34, CD11b, CD19, CD45 and MHC Class II) antibodies and assayed as per manufacturer's instructions (hMSC Analysis Kit, 562245, BD Biosciences©), using a BD FACSCalibur™ 3 CA Becton Dickinson (BD Biosciences©). Flow cytometry data was processed using FlowJo Engine X10.4 (v3.05478, LLC).

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Gene expression. Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR) were performed for MSCs' related genes sequences from Bio Rad®: CD34 (qHsaCID0007456), CD90/THY1 (qHsaCED0036661), CD73/NT5E (qHsaCID0036556), CD105/ENG (qHsaCID0010800), CD166/ALCAM (qHsaCID0037887), CD117/c-kit (qHsaCID0008692), SOX2 (qHsaCED0036871), OCT3-4/POU5F1 (qHsaCED0038334), MHC Class I/HLA-A (qHsaCED0037388), MHC Class II/HLA-DRA (qHsaCED0037296) and housekeeping genes: β-actin (qHsaCED0036269), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (qHsaCED0038674).

Cultured hDPSCs in P5–P7 were harvested using Trypsin-EDTA (25200072, Gibco®), and pellets of 1×10^6 cells of each group were used for total RNA extraction, using the Aurum™ Total RNA Mini kit (732-6820, Bio Rad®) [31]. Briefly, cell pellets were lysed, DNA was removed with DNase I enzyme and obtained RNA was eluted. Total RNA was quantified using nanophotometer readings at 260 and 280 nm (NanoPhotometer™ Pearl, Implen GmbH). Once RNA concentrations were tuned between samples, cDNA was synthesized from the purified RNA using the iScript Reverse Transcription kit (170-8891, Bio Rad®) and T100 Terma Cyclar Thermocycler (Bio Rad), as per manufacturer's instructions. qPCR was performed in a CFX96 Touch™ (BioRad®) apparatus using the iTAQ™ SYBR® Green Supermix (172-5120, BioRad®) and custom PCR plates encompassing duplicates of targeted human genes and a negative control. Recommended PrimePCR cycling protocol was employed: 95° C for 2 min (activation), 40 cycles comprising 95° C for 5 s (denaturation), −60° C for 30 s (annealing), and 65–95°C (0.5°C increments), 5 s/step (melt curve). The number of cycles for each well was recorded. Data was processed using BioRad CFX® Manager Software 3.1 (Bio Rad Laboratories). Fold differences were calculated using the standard ΔCq method with GAPDH and β-actin as housekeeping genes.

Multi-lineage differentiation. hDPSCs (P5-P7) were plated onto 24-well plates (8.000 viable cells/cm^2) and cultured in standard media until reaching a confluency of 70–80% of culture surface. Cells were transitioned onto specific differentiation media for adipogenesis (StemPro® Adipogenesis Differentiation kit, A10070-01, Gibco®) and osteogenesis [standard culture medium supplemented with 5 nM Dexamethasone (D8893 – Sigma Aldrich®), 250 μM ascorbic acid-2-phosphate (A4403, Sigma Aldrich®) and 10 mM β-glycerphoshate (G9422, Sigma Aldrich®)]. Undifferentiated controls were maintained in standard media. Media were refreshed every 3 days, for 21 days. After 14 days, cells under adipogenic differentiation were stained with Oil Red O (ORO), for lipid droplet detection. ORO stain was solubilized using 100% isopropanol (59300, Merck Milipore®), and its absorbance quantified at 570 nm.

Von Kossa staining was employed to visualize mineral deposition. Cells were fixated and dehydrated with sequentially increasing ethanol concentrations and let dry. Cells were rehydrated and incubated with 2% Silver nitrate solution (85193, Sigma Aldrich®), under Ultra-violet light. Sodium thiosulfate 5% (72049, Sigma Aldrich®) was added and incubated for 3 min. Wells were rinsed and photographic record obtained. Alizarin Red S (ARS) assay was used to determine osteogenic differentiation of hDPSCs, as previously described in [31]. After fixation with 4% formaldehyde, cells were stained with 40 mM ARS (2003999, Milipore®). Following a 30-min incubation, unbound dye was removed and ARS was extracted for quantification, with 10% acetic acid (ARK2183, Sigma-Aldrich®). Individual absorbance values were measured at 405 nm through the interpolation of standard curve of ARS concentration (μM).

For chondrogenic differentiation, hDPSCs were plated at 2×10^4 viable cells/well onto a 96-well plate. After 48 h, cells were transferred to chondrogenic culture medium (StemPro® Chondrogenesis Differentiation kit, A10071-01, Gibco®) and control wells remained in standard supplementation. Media were renewed every other day for 14 days. After 14 days, Proteoglycan's synthesis by differentiated chondrocytes was assessed though Alcian Blue staining. Cells were fixated with 4% formaldehyde (252931.1315, Panreac® AppliChem), and stained with Alcian Blue (A9186, Sigma®) solution. Alcian Blue was removed and cells were rinsed with acetic acid at 3% (v/v)(A6289, Sigma Aldrich®). Acidity was neutralized with distilled water. Differentiated cells were observed under inverted microscope. Sulphated glycosaminoglycan (GAGs) production was determined to assess chondrogenic matrix production, following manufacturers' instructions. GAGs were extracted using Papain (P3125, Sigma-Aldrich®), and Blyscan™ dye reagent (Glycosaminoglycan Assay Blyscan™, Biocolor) was used to induce the precipitation of extracted compounds. Precipitate was solubilized and absorbance at 656 nm was recorded, along with a standard curve for the interpolation of GAG concentration (μg/ml).

In vivo bone regeneration assessment

All the animal testing procedures were in conformity with the Directive 2010/63/EU of the European Parliament and the Portuguese DL 113/2013. All the procedures were approved by the ICBAS-UP Animal Welfare Organism of the Ethics Committee and by the Veterinary Authorities of Portugal. Humane end points were followed in accordance to the OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000), and adequate measures were taken to minimize pain and discomfort considering human endpoints for animal suffering and distress. The interaction of Bonelike® in bone tissue was evaluated in 12 healthy skeletally-mature Merino sheep (*Ovis aries*) with an average weight of ∼50 kg and aged between 7 and 8 years.

Surgical procedure

Pre-surgical procedure. Multimodal anaesthesia was employed, and premedication was administered intramuscularly into the lumbar muscles using xylazine (Rompun® 20 mg/ml, Bayer, 0.1 mg/kg), together with butorphanol (Dolorex® 10 mg/ml, MSD, 0.05 mg/kg). Initial sedation allowed trichotomy of the hind limb. Initial antisepsis of the surgical site was performed with 4% iodopovidone (Betadine®) scrub solution. Intraoperative intravenous fluid therapy (NaCl 0.9% B Braun®) at a maintenance rate was provided. Induction of general anaesthesia was performed by intravenous bolus administration of tiletamine-zolazepam (Zoletil®100, Virbac, 5.5 mg/kg). Loco-regional anaesthesia was provided using lidocaine hydrochloride (Anestesin® 2%, Medinfar-Sorológico) administered via an epidural spinal catheter. Final antiseptic preparation of the operative field was performed with the animal in lateral recumbency using 70% ethanol (Aga) and 10% iodopovidone topical solution. Anaesthetic monitoring of cardiorespiratory parameters was performed and recorded, while intravenous top-up bolus of tiletamine-zolazepam was administrated intraoperatively whenever required.

Surgical technique. An ∼15 cm skin incision was made lateral to the diaphysis of the femur, from proximal to distal, and the lateral

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diaphysis of the femur was exposed. In the lateral diaphysis, a row of five holes with a non-critical size defect of 5.0-mm diameter was drilled through the cortex and into the medulla using a micro-burr (HG.28 Handpiece coupled with an M-SR-FCT motor, Foredom®). Continuous flushing with 0.9% sterile saline solution was performed during drilling to minimize thermal damage and to remove any residual bone fragments. A minimal distance of 1 cm was kept between drilled holes to reduce the risk of iatrogenic fracture, and to allow multiple serial analyses of the biomaterial in the same *in vivo* conditions.

Biomaterial and cellular system application. The bone defects were assigned to either untreated (control), biomaterial (Bonelike®) associated with Tisseel Lyo®(Baxter), or Bonelike® *plus* Tisseel Lyo® and hDPSCs study groups. Bonelike® granules were combined with Tisseel Lyo® to help retaining the biomaterial and hDPSCs within the defect, working as a cellular-based therapy vehicle. Tisseel Lyo® is a fibrin glue of human origin, comprised of fibrinogen and thrombin and the resultant fibrin clot is very similar to that of normal blood coagulation. It is completely biodegraded by the host's normal anti-fibrinolytic pathways and does not induce any systemic or local rejection reaction. In the cell-treated defects, hDPSCs (obtained by *in vitro* culture, as described above) were homogenously incorporated in the Bonelike® and Tisseel Lyo® mixture at a cell density of 10×10^4 cells per defect.

Post-surgical procedures. Post-operative radiographic follow-up was performed to assess and monitor the surgical defects. After recovery from anaesthesia, animals were transferred to an individual feeding cage for 24 h in order to minimize movements immediately after the surgery. Afterwards they were transferred to a straw yard, with two or three other animals. The intramuscular antibiotic Pendistrep® (6000 IU/kg of benzylpenicillin procaine and 7.5 mg/kg of dihydrostreptomycin, Syva®) and a non-steroidal anti-inflammatory drug (Carprofen-Rimadyl®, 50 mg/ml, Zoetis, 0.7mg/kg) were given post-operatively and daily for 5 days. To assess for *in loco* bone regeneration phenomena, animals were sacrificed at different time points as described below. This was accomplished in a quiet and isolated environment, under humane conditions using an overdose of intravenous 20% sodium pentobarbital (Eutasil®, 200 mg/ml, Ceva). The time-points for the healing process analysis were 30, 60 and 120 days post-operatively, using four animals for each experimental group. Short periods (30 and 60 days) were chosen to observe the initial phase of bone healing, and the longer period (120 days) was performed to allow analysis of the more advanced stages of bone healing, as well as the absorption and degradation of the biomaterial. *Post-mortem*, femurs were dissected and soft tissue was removed from the bone. Radiographic imaging of the collected bones was immediately performed and samples were preserved in 4% formaldehyde. The femur was sectioned to detach each of the five defect sites separately and the samples were subsequently evaluated by histomorphometric and histological analysis.

Histological processing and histomorphometric analysis

Bone segments were immersed in increasing percentage alcohol solutions (from 70 to 99%) and finally embedded in a Methyl Methacrylate resin (Merck KGaA, Germany). Using a microtome (Accutom, Struers, Denmark) with a diamond blade (TAAB, Griding Wheel Institute, OH, USA), sections were cut in transverse to 150-µm wide and hand-ground to ~70–80 µm. Sections were then stained with Solochrome Cyanine R (Merk) for histological examination using light microscopy (Eclipse E600, Nikon, Tokyo, Japan), equipped with a calibrated digital camera (Nikon DS-5M-L1 Digital Sight Camera System, Nikon). Unstained slides were used to perform SEM analysis. The SEM and energy dispersive x-ray spectroscopy (EDS) analysis were performed using a high-resolution (Schottky) environmental SEM with X-ray microanalysis and electron backscattered diffraction (Quanta 400 FEG ESEM/EDAX Genesis X4M), operating in high vacuum mode at an accelerating voltage of 15 kV.

According to the technique developed by Atayde et al. [32] the obtained images were divided into three fractions, namely: (i) the periosteal callus (zone of new bone formation over the defect area); (ii) the defect area (square area with its vertices intercepting the defect wall); (3) the endosteal callus (zone of new bone formation underneath the defect area) (Supplementary Fig. S1(1)). Histological images were analysed to evaluate the cellular reaction and the quantity of new bone formation.

Threshold analysis

The Threshold analysis technique was optimized in Atayde et al. [6, 32], and herein employed to quantitatively monitor and evaluate bone and biomaterial interactions at the determined time points (30, 60 and 120 days). Briefly, grey scale images obtained from unstained sections were uploaded to the Image J® software (https://imagej.nih.gov/ij/), converted to 8-bit and run through the Threshold tool (Image/Adjust/Threshold). Maximum and minimum threshold values were defined to separate and measure fraction areas of the lacunae/unfilled zone, new bone and biomaterial (Supplementary Fig. S1(2))). Finally, pergentage (%) of new bone [(defect new bone area/defect total area*100], Bonelike® [(defect Bonelike® area/defect total area*100], Unfilled area [(defect Unfilled area/defect total area*100], and Lacunae area [(defect Lacunae area/defect total area*100]. Bone growth at different times post-implantation was also assessed in the different fractions (defect area, periosteal callus and endosteal callus).

Statistical analysis

Statistical analysis was performed using GraphPad Prism® (version 6.00 for Mac OS X, GraphPad Software, La Jolla, CA, USA). Results were presented as mean ± standard error of the mean (SEM). Comparisons between groups were performed by one-way analysis of variance followed by Tukey's multiple comparisons test. Differences were considered statistically significant when $P \leq 0.05$. Significant results between groups were presented using the symbol (*). Significance results are also indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 < P \leq 0.05$, $0.001 < P \leq 0.01$, $0.0001 < P \leq 0.001$ and $P \leq 0.0001$, respectively.

Results

Bonelike® SEM

SEM was employed to visualize prepared Bonelike® granules, and detail on its morphology and topography (Fig. 1).

In Fig. 1A the spherical structure of the Bonelike® is observed. At higher magnification, it is possible to visualize a morphology with different roughness levels (Fig. 1B) and the presence of microporosity, homogeneously distributed over the entire sphere (Fig. 1C).

DPSC characterization

DPSCs were demonstrated to present characteristic hMSCs' markers, as assessed through flow cytometry (Fig. 2A). Over 90% of the population

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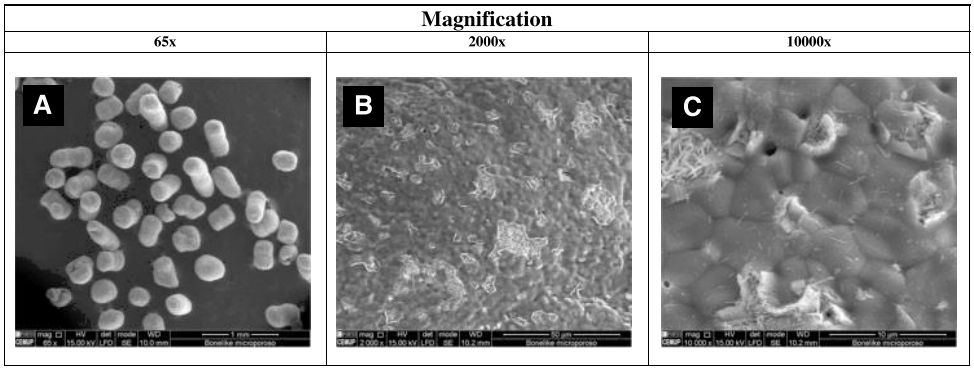


Figure 1. SEM images of Bonelike® granules (Total magnification: **(A)** 65×, **(B)** 2000× and **(C)** 10 000×)

was positive for CD90, CD105 and CD44, and ≤ 2% were negative for CD34, CD11b, CD19, CD45 and MHC II.

Gene expression was performed through RT-qPCR analysis. Total RNA was successfully extracted from cultured hDPSCs and specific gene expression was assessed (Fig. 2B). CD34 was not detected as expected for hDPSCs. CD105, CD73 and CD90 were highly expressed; CD166, MHC I and CD117 showed strong to moderate expression. Multipotency genes as Nanog, Oct4, Sox2, were also weakly expressed (CT value > 35). Moreover, weak expression of MHC Class II was detected in hDPSCs by RT-qPCR analysis, however, membrane expression demonstrated by flow cytometry, was not detected [33]. Tri-lineage differentiation was quantitatively evaluated through ORO, ARS and GAGs protocols, to evaluate adipogenic, osteogenic and chondrogenic differentiation, respectively. Results demonstrated successful differentiation towards the three lineages, with significant differences from undifferentiated controls (Fig. 2C and D).

In vivo bone regeneration assessment

Radiologic study

Radiographs were obtained in craniocaudal and mediolateral projections of the femur at the different times of implantation (30, 60 and 120 days). Bone defects left untreated (Fig. 3A), treated with Bonelike® (Fig. 3B) and Bonelike® *plus* hDPSCs (Fig. 3C–E) are identifiable in Fig. 3.

At the earliest study timepoint (30 days) the defects treated with Bonelike®, and Bonelike® *plus* hDPSCs, (Fig. 3(1-2), B–E) showed a radiopaque central circle with a radiolucent edge. In the untreated controls, only radiolucent circles were visible (Fig. 3(1-2), A). The radiolucent edge around the defects containing the biomaterial suggests an initial phase of bone regeneration, where osteoclasts remove debris from the bone edges, leading to increased areas of reabsorption [34]. Over time, the radiolucent edges decrease until totally disappearance, representing reabsorption impairment and triggered regeneration (Fig. 3(3-4), A–E). Complete attenuation of the radiolucent edge was seen at 120 days post-implantation, clearly reflecting the transition from the reabsorption phase to the repair phase during the healing process. After 120 days, defects treated with the hDPSCs and Bonelike® (Fig. 3(5-6), C–E), showed significant radiographic evidence of improved bone regeneration, compared with other groups at this healing period.

Histological analysis

SEM images were obtained from histological section of the bone samples at 30, 60 and 120 days post-implantation (Fig. 4).

At the earliest time of analysis, the bone defect sites were easily identified, presenting sharp edges in all study groups. In the Bonelike® treated groups, the biomaterial was visualized inside the defect, presenting slight to moderate protrusion into the medullary canal (as evidenced in Fig. 4B and C). The degree of protrusion of Bonelike® into the medullary canal presented great variability between individuals, especially at 30 days post-implantation. At longer time points, this effect was attenuated.

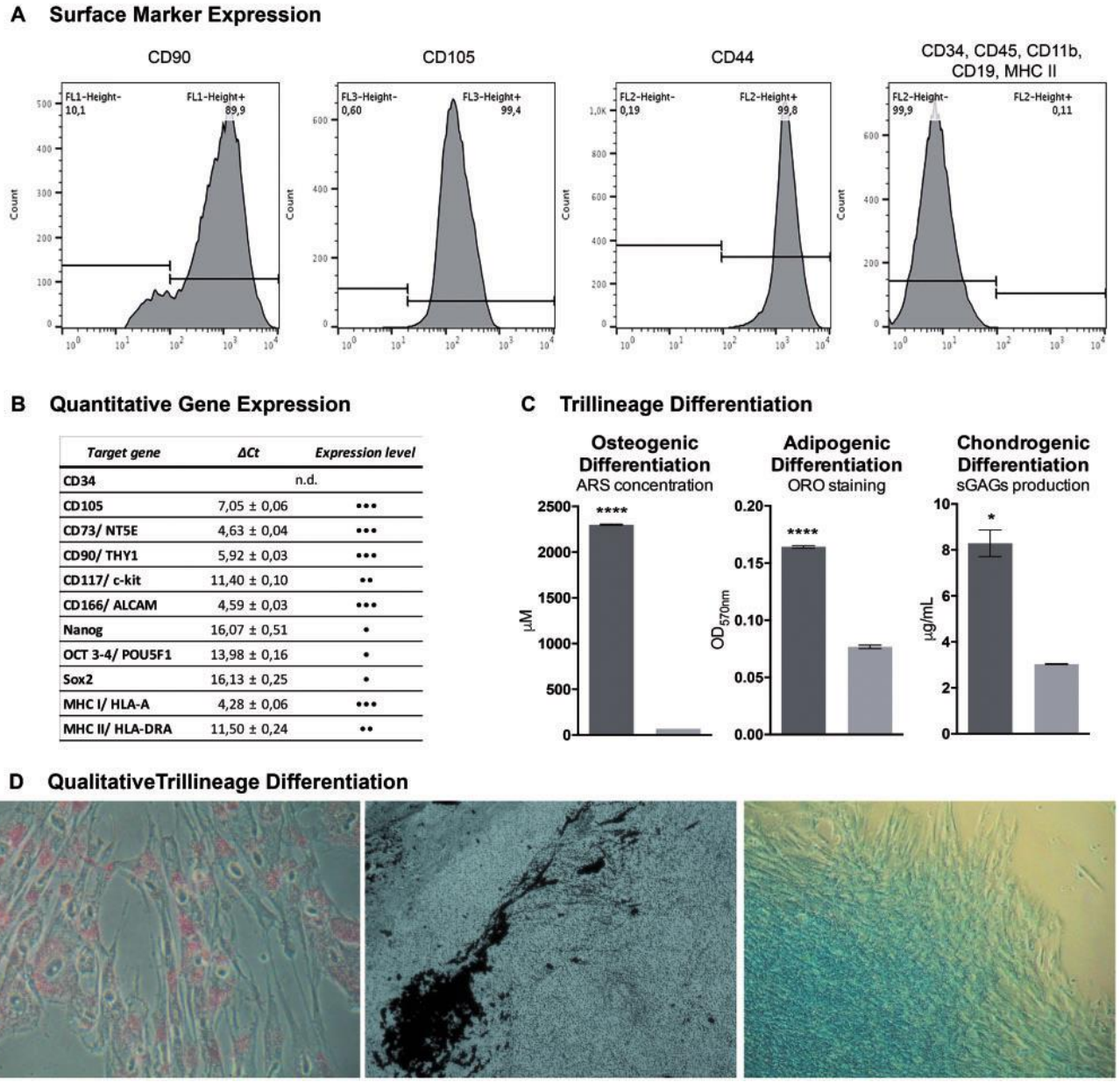
At 60 days post-implantation, the defect edges were softened in all groups, with new tissue formation towards the centre of the defect. In the untreated control and Bonelike® treated groups the newly grown tissue presented an organization distinguishable from the surrounding native bone (Fig. 4D and E, respectively). Contrarily, the groups including hDPSCs within the biomaterial system presented structural features closer to those of the surrounding healthy bone (Fig. 4F). Complete bridging of the opposing edges of the bone defect was only observable in the Bonelike® *plus* hDPSCs treated group, at this time period.

At the final 120 days of bone regeneration assessment, the control group samples achieved partial bridging of the defect on the central area of the defect, while the endosteal and periosteal margins remained unbridged (Fig. 4G). Bone resorption *loci* are observable as empty areas within the newly formed tissue. On the other hand, the Bonelike® and Bonelike® *plus* hDPSCs treated groups were completely bridged, with the new tissue completely enclosing the bonelike granules. Periosteal and endosteal surfaces continuity was completely restored in both groups (Fig. 4H and I).

Higher magnification SEM imaging enabled the visualization of further details, focussing on the biomaterial–tissue interface. Initially, an increase in porosity was noted in the biomaterial–bone interface, with the formation of voids within the particles (Fig. 5A). With increasing time post-implantation, these voids were replaced by mineralized tissue (Fig. 5B and C). Thus, it was possible to observe the inclusion of Bonelike® in the area of new bone and its presence within newly formed gaps (osteointegration), without evident adverse effects on the surrounding tissue (Fig. 5C). The spherical shape and the granulometry of Bonelike® appeared to be atraumatic to the surrounding hard tissues, and to naturally adapt to the circular pattern of bone growth.

EDS analysis (Supplementary Fig. S2) was performed in order to address the chemical composition of the material within the biomaterial with the newly formed bone, mature bone and Bonelike® itself. EDS analysis revealed a peak of sodium (Na^{+}) and magnesium (Mg^{2+}) within the biomaterial granules, characteristic of old and newly formed bone.

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Target gene	*ΔCt*	*Expression level*
CD34	n.d.	
CD105	7,05 ± 0,06	•••
CD73/ NT5E	4,63 ± 0,04	•••
CD90/ THY1	5,92 ± 0,03	•••
CD117/ c-kit	11,40 ± 0,10	••
CD166/ ALCAM	4,59 ± 0,03	•••
Nanog	16,07 ± 0,51	•
OCT 3-4/ POU5F1	13,98 ± 0,16	•
Sox2	16,13 ± 0,25	•
MHC I/ HLA-A	4,28 ± 0,06	•••
MHC II/ HLA-DRA	11,50 ± 0,24	••

Figure 2. hDPSCs characterization. **(A)** Surface marker expression for hDPSCs identity, assessed by flow cytometry; **(B)** Quantitative gene expression of hMSCs and pluripotency markers, by RT-PCR (•, weak; ••, moderate; •••, strong expression); **(C)** Osteogenic, adipogenic and chondrogenic differentiation determined by ARS, ORO and sulphated GAGs quantification (Significance results are also indicated according to *P* values with one, two, three or four of the symbols (*) corresponding to $0.01 < P \leq 0.05$, $0.001 < P \leq 0.01$, $0.0001 < P \leq 0.001$ and $P \leq 0.0001$, respectively); **(D)** Adipogenic, osteogenic, and chondrogenic differentiation visualized through ORO, von kossa and alcian blue histochemical staining

Histological sections were stained with Solochrome Cyanine R, to allow bone tissue visualization and remodelling assessment.

At 30 days post-implantation, untreated control sections presented very small amounts of newly formed bone, that was predominantly of immature nature (woven and trabecular bone) (Fig. 6A). Contrariwise, in the defects treated with the biomaterial, a higher proportion of compact new bone with a mature lamellar configuration was observed. Furthermore, Haversian systems were observed (Fig. 6B and C). Even as early as 30 days post-implantation, these sections showed good osteointegration, with new bone opposed to the granules and encircling osteocytes. As post-implantation time progressed, bone ingrowth became more evident (Fig. 6D–I); also the formation of Haversian systems and lamellas encircling and encroaching on the Haversian canals are observed.

Bonelike® and Bonelike® *plus* hDPSCs-treated defects demonstrated good osteointegration, with the formation of new bone around biomaterial particles, in early implantation times. At 30 days post-implantation, initial absorption events were observed, with a low degree of biomaterial degradation, while at 120 days post-implantation it was possible to observe a high absorption and degradation rate, and biomaterial spheres contour alteration.

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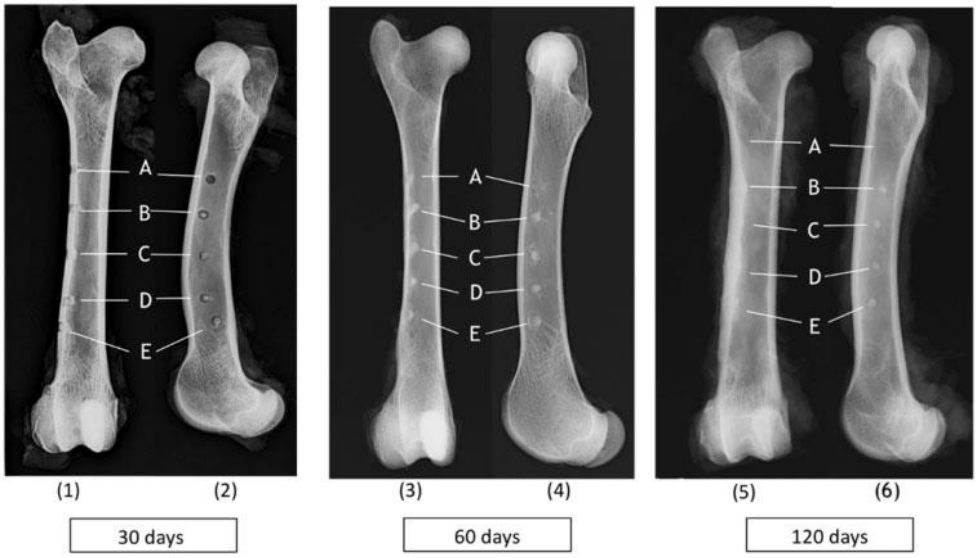


Figure 3. Radiographic images [craniocaudal (1, 3, 5) and mediolateral (2, 4, 6) views] of ovine femurs showing bone regeneration after 30, 60 and 120 days post-implantation of **(A)** untreated control; **(B)** Bonelike®; **(C–E)** Bonelike® *plus* hDPSCs

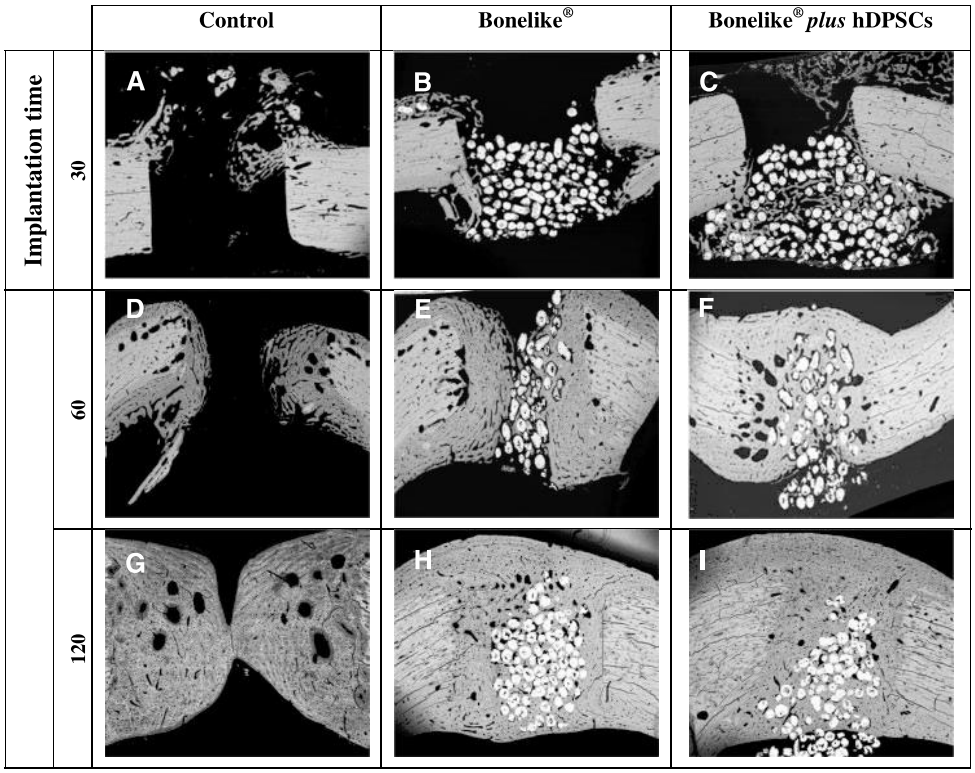


Figure 4. SEM of bone sections representative images from control, Bonelike® and Bonelike® *plus* hDPSCs groups at each implantation time (amplification: 30×)

Histomorphometric analysis

Bone sections submitted to histological processing were further analysed histomorphometrically to evaluate the osteo-integration, osteoconduction and degree of absorption on the studied groups, through semi-automatic image segmentation.

At all times (30, 60 and 120 days), the results demonstrate that samples treated with the addition of the hDPSCs system depicted increased new bone formation. A greater increase in new bone is observed from 30 to 60 days, when compared with 60–120 days (Fig. 7A) [30 days: untreated control (8.6%), Bonelike® (13.1%) and Bonelike® *plus* hDPSCs (15.2%); 60 days: untreated control (45.3%), Bonelike® (48.5%) and Bonelike® *plus* hDPSCs (59.4%); 120 days: untreated control (62.6%), Bonelike® (67.9%), and Bonelike® *plus* hDPSCs (77.5%)].

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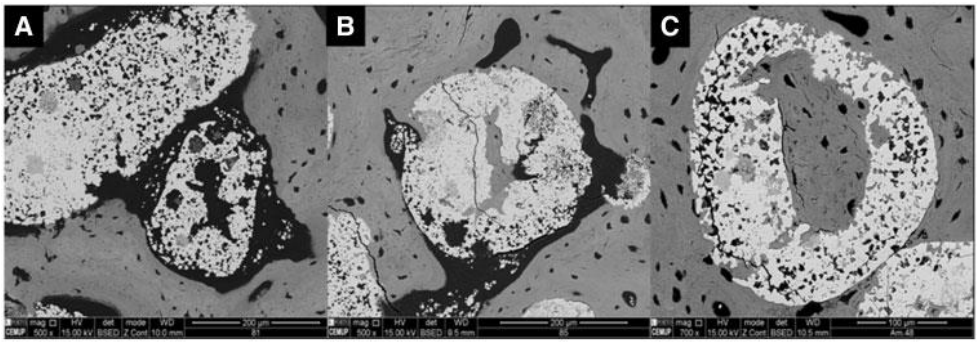


Figure 5. Neo-formed bone inside Bonelike® by SEM analysis of bone defects after 30, 60 and 120 days of implantation. (amplification: **A** and **B**: 500×, **C** 700×). White, —Bonelike®; grey, neo-formed bone

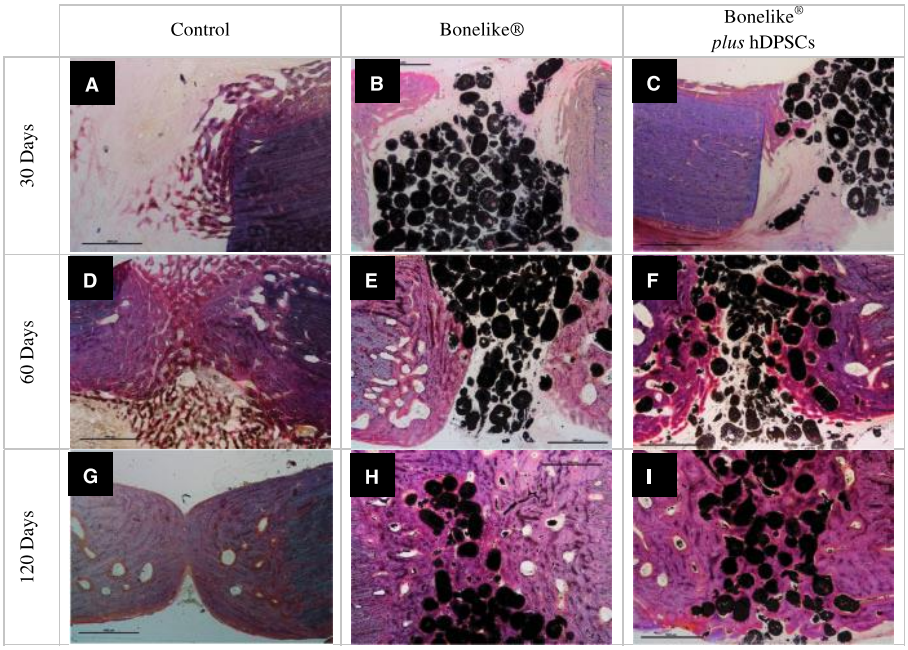


Figure 6. Solochrome cyanine R staining of bone defects sections (control, Bonelike® And Bonelike® *plus* hDPSCs), at 30, 60 and 120 days post-implantation. Samples were stained with solochrome cyanine R to differentiate osteoid from newly deposited bone and older bone. Black, Bonelike® spheres; blue, mature bone; pink, neo-formed bone (Amplification 20×)

The degree of reabsorption of the biomaterial was quantified by comparing the variation of the area occupied by the biomaterial throughout time regarding the different groups (Fig. 7B). Results show that the amount of Bonelike® decreased over time post-implantation, as it was gradually replaced by new bone, confirming its biodegradable properties. Although not statistically different, defects treated with biomaterial alone showed a tendency to maintain higher content of Bonelike® when compared with the hDPSCs inclusive formulation, suggesting decreased biomaterial reabsorption. By analysing the percentage of unfilled area (Supplementary Fig. S3) it is evident that biomaterial-treated defects showed higher percentage of filled area, than controls (Supplementary Tables S1–S3).

Importantly, at 120 days post-implantation, the control sections still had unfilled areas, whereas the treatment groups did not. Indeed, control groups showed a higher fraction of lacunae compared with treatment groups, which indicate increased bone fragility in the first. Supplementary Fig. S4 shows an analysis of new bone formation of the different bone areas (periosteal *callus*, defect area and endosteal *callus*). At 30 and 60 days post-implantation, there is a tendency for a greater production of (immature) bone in biomaterial treated defects (Supplementary Tables S4 and S5). At 120 days post-implantation, there was no variation in bone healing between the three zones (Supplementary Table S6); at this time the bone is deemed completely ossified.

Discussion

Demographic factors, such as the increase in the average life expectancy of worldwide population, have breached the demand for efficient strategies for bone disease therapy [1, 2]. Although bone tissue transplant (grafting) has been the option of choice for severe fractures therapy [3, 4], it still presents relevant limitations, boosting the need for the alternatives, such as synthetic bone substitutes.

Herein, we complemented the work developed by Atayde et al. [6] that demonstrated the osteointegration, osteoconduction and

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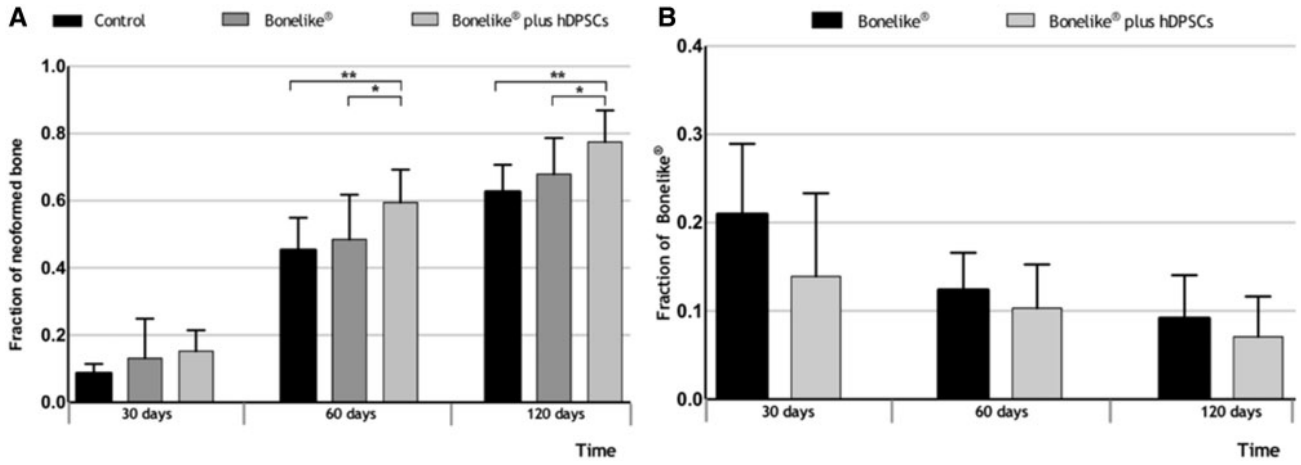


Figure 7. Histomorphometric analysis of biomaterial-bone interface in the defect area. Results presented as mean ± SD of the neo-formed bone **(A)** and Bonelike® **(B)** fractions at defect area in the control, Bonelike® and Bonelike® *plus* hDPSCs groups, at 30, 60 and 120 days post-implantation (*$0.01 < P \leq 0.05$; **$0.001 < P \leq 0.01$)

degradation dynamics of the synthetic bone substitute Bonelike®, by adding a cellular component to improve the regenerative system.

hDPSCs were selected due to their accessibility and availability throughout life. Further, this specific population presents a tendency towards the osteogenic lineage and has been demonstrated to endure mineral deposition both *in vitro* and *in vivo* [35]. The hDPSCs system herein employed demonstrated to fully comply with the ISCT's recommendations for the characterization of MSCs population [13]. The population presented negative to CD34 surface marker. This marker is found expressed in primitive pluripotent stem cells, of both stromal and haematopoietic origin, and is commonly regarded as a haematopoietic marker for stem cells populations. Although most authors confirm the $CD34^-$ phenotype, $CD34^+$ DPSCs populations have been identified and deemed to retain other MSC characteristics. These were further demonstrated to present enhanced bone formation capacity [36, 37].

Gene expression analysis indicated weak expression of MHC Class II expression, but it did not reflect the effective presence of the molecule in the cellular membrane, as demonstrated by flow cytometry [33]. This weak to absent expression of HLA-DR surface molecules supports the allogenic and xenogenic application of these cells, with reduced risk of immune rejection by the receptor and the need of immunosuppression [38], as observed in this experiment. Also, the proposed system demonstrated great efficiency in osteodifferentiation under *in vitro* specific stimuli. The mineralogenic tendency of hDPSCs is supported by their early expression of putative bone-marker (even in primary undifferentiated isolates), as well as their inherent tendency for spontaneous differentiation [39, 40].

Furthermore, hDPSCs have been demonstrated to secrete an extensive array of bioactive factors (growth factors and cytokines) [30] that modulate the activity of native cells and inflammatory populations at lesion sites and therefore unleash the regenerative response.

To assess for the regenerative potential of the synthetic biomaterial Bonelike® in combination with hDPSCs, a standardized non-critical pre-clinical bone defect model was employed. The ovine model was selected due to its phylogenetic proximity to the human species, regarding its musculoskeletal size and biomechanics. Further, bone tissue of both species presents histological similarities, such as the presence and structure of *Havers* channels and the process of cortical remodelling of bone structure [16].

Bone regeneration and remodelling of untreated and treated defects was monitored over 30, 60 and 120 days. Macroscopic radiographic assessment indicated the presence of the biomaterial at the defect site, surrounded by a radiolucent edge. The radiolucent edge around the defects containing the biomaterial suggest an initial phase of bone regeneration, where osteoclasts remove debris from the bone edges, leading to increased areas of reabsorption [34]. Over time, the radiolucent edges decrease until totally disappearing, representing reabsorption impairment and triggered regeneration. Complete attenuation of the radiolucent edge was seen at 120 days post-implantation, clearly reflecting the transition from the reabsorption phase to the repair phase during the healing process. After 120 days, defects treated with the hDPSCs and Bonelike® showed significant radiographic evidence of improved bone regeneration, compared with other groups at 120 days of healing period.

The signs of bone regeneration were further confirmed microscopically. As has been reported, one of the challenges to granular biomaterial application is its maintenance at the lesion/delivery site. Although we associated a fibrin glue component to the Bonelike® granules, we still observed variable degrees of system dislodgement towards the bone marrow cavity. The biomaterial was verified on histology to be within the defect margins, although the existence of some material in the medullary zone was recorded. Also, we observed some degree of inter-subject variability regarding new bone formation reinforcing the need for careful planning of experimental procedures and adequate number of animals per experimental group.

Accelerated defect bridging in the biomaterial filled defects was observed. The biomaterial posed as both a stimuli and scaffold of newly-formed tissue penetration and is progressively embedded within the newly deposited matrix. The observed features confirm other authors previous findings [6]. Biomaterial-treated defects showed faster bone maturation than controls; with lamellar bone formation and arise of Haversian systems. This phenomenon reflects the osteopromotion associated to the porous nature of the biomaterial, which facilitates cell anchoring and protein absorption and consequently better osteoconduction. Indeed, the microtomography (roughness) of the biomaterial has been demonstrated to be of the utmost importance to hDPSCs adhesion and osteopromotion [41], and that microconcave surfaces favour osteodifferentiation and formation of thick mineralized tissue by seeded cells, due to the resemblance to native bone trabecular spaces [42]. The spherical shape

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and the granulometry of Bonelike® have been previously identified as to naturally adapt circular pattern of native bone growth [6]. These features also enhance cell migration, proliferation of native and delivered cells, as well as improving vascular invasion, resulting in optimized osteointegration and osteo-reabsorption.

The addition of hDPSCs to the therapeutic system resulted in higher bone growth and accelerated defect bridging, ensuring contact of bone edges from the centre to the endosteal surface of the bone. Immunohistochemical staining also denoted increased mature lamellar bone content in Bonelike® treated defects, with improved histological features recovery. The improvement and acceleration of bone regeneration events is particularly evident at the earliest times of regeneration.

Biomaterial reabsorption is neglectable at the first observation time but becomes evident after 4 months. The reabsorption phenomena are indicated by the outline attenuation and bone tissue invasion inside voids created within the biomaterial granules. Reabsorption phenomena occurring mainly by dissolution and enzymatic/cellular activity (osteoclasts), release mineral elements that are re-incorporated in the newly formed bone. The hDPSCs inclusive formulation, tendentially presented inferior Bonelike® content when compared with the cell-free formulation, suggesting increased biomaterial reabsorption rates.

The combination of mineral and non-mineral based biomaterials in association to dental-derived MSC populations aiming at bone regeneration is regarded as an enthusiastic road for therapy development, as revised in [35]. The proposed combinations unanimously provided improved accelerated and increased bone formation, of predominant mature lamellar nature, finally resulting in remodelled bone with near-native histological features [43]. Comparable observations resulted from the presently proposed combination of Bonelike® and hDPSCs.

Conclusions

This study describes the *in vivo* performance of Bonelike® in association to hDPSCs in an ovine model of non-critical bone defects at 30, 60 and 120 days post-implantation. It demonstrated Bonelike® associated with hDPSCs to be a very promising therapeutic system for bone regeneration. Results obtained by post-mortem bone radiology besides histological and histomorphometric analyses show that defects filled with Bonelike® and hDPSCs had overall superior new bone formation.

The study of this system's performance in non-critical bone defects is an essential step to its progression towards clinical applications from small defects (such as alveolar defects resulting from dental extractions) up to extensive trauma events (currently addressed through auto-, allo- and xenogeneic bone transplantation). Further studies ought to entail on this system's performance in increasing defect sizes, aiming at their effective application on clinical scenarios. We envision their inclusion in non-critical fractures treatment, to accelerate and improve regeneration as well enabling the healing of critical and/or non-healing defects.

Supplementary data

Supplementary data are available at *REGBIO* online.

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Conflict of interest statement. None declared.

References

1. European C. *Demography Report - Short Analytical Web Note*. Luxembourg, 2015, 6–19.
2. Bohner M. Resorbable biomaterials as bone graft substitutes. *Mater Today* 2010;**13**:24–30.
3. Wang W, Yeung KWK. Bone grafts and biomaterials substitutes for bone defect repair: a review. *Bioactive Mater* 2017;**2**:224–47.
4. Campana V, Milano G, Pagano E *et al*. Bone substitutes in orthopaedic surgery: from basic science to clinical practice. *J Mater Sci Mater Med* 2014;**25**:2446–61.
5. Salgado AJ, Coutinho OP, Reis RL. Bone tissue engineering: state of the art and future trends. *Macromol Biosci* 2004;**4**:743–65.
6. Atayde LM, Cortez PP, Afonso A *et al*. Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike®) on osteoregeneration. *J Biomed Mater Res Part B Appl Biomater* 2015;**103**:292–304.
7. Hussain NS, Lopes MA, Maurício AC *et al*. Bonelike® graft for regenerative bone applications. *Surface Engineered Biomedical and Surgical Devices* 2007;**13**:477–512.
8. Morais DS, Rodrigues MA, Silva TI *et al*. Development and characterization of novel alginate-based hydrogels as vehicles for bone substitutes. *Carbohydr Polym* 2013;**95**:134–42.
9. Jha LJ, Santos JD, Knowles JC. Characterization of apatite layer formation on P2O5-CaO, P2O5-CaO-Na2O, and P2O5-CaO-Na2O-Al2O3 glass hydroxyapatite composites. *J Biomed Mater Res Part A* 1996;**31**:481–6.
10. Santos JD, Jha LJ, Monteiro FJ. In vitro calcium phosphate formation on SiO2-Na2O-CaO-P2O5 glass reinforced hydroxyapatite composite: a study by XPS analysis. *J Mater Sci Mater Med* 1996;**7**:181–5.
11. Knowles JC, Hastings GW, Santos JD. *Sintered hydroxyapatite compositions and method for the preparation thereof*. In: UC London, (ed). England, 2000. International patent (2000) WO/2000/068164.
12. Biosckin SA. MaCT. Bonelike. In. www.biosckin.pt.
13. Dominici M, Le Blanc K, Mueller I *et al*. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–7.
14. Gronthos S, Brahim J, Li W *et al*. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002;**81**:531–5.
15. Caseiro AR, Pereira T, Bártolo PJ *et al*. Mesenchymal stem cells and biomaterials systems – perspectives for skeletal muscle tissue repair and regeneration. *Proc Eng* 2015;**110**:90–7.
16. Martini L, Fini M, Giavaresi G *et al*. Sheep model in orthopedic research: a literature review. *Comp Med* 2001;**51**:292–9.

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17. Alexandre N, Amorim I, Caseiro AR *et al*. Long term performance evaluation of small-diameter vascular grafts based on polyvinyl alcohol hydrogel and dextran and MSCs-based therapies using the ovine pre-clinical animal model. *Int J Pharm* 2017;**523**:515–30.
18. Alexandre N, Costa E, Coimbra S *et al*. In vitro and in vivo evaluation of blood coagulation activation of polyvinyl alcohol hydrogel plus dextran-based vascular grafts. *J Biomed Mater Res A* 2015;**103**:1366–79.
19. Alexandre N, Ribeiro J, Gartner A *et al*. Biocompatibility and hemocompatibility of polyvinyl alcohol hydrogel used for vascular grafting–In vitro and in vivo studies. *J Biomed Mater Res A* 2014;**102**:4262–75.
20. Caseiro AR, Pereira T, Ivanova G *et al*. Neuromuscular regeneration: perspective on the application of mesenchymal stem cells and their secretion products. *Stem Cells Int* 2016;**2016**:1.
21. Gartner A, Pereira T, Alves MG *et al*. Use of poly(DL-lactide-epsilon-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly of the umbilical cord for promoting nerve regeneration in axonotmesis: in vitro and in vivo analysis. *Differentiation* 2012;**84**: 355–65.
22. Gartner A, Pereira T, Simoes MJ *et al*. Use of hybrid chitosan membranes and human mesenchymal stem cells from the Wharton jelly of umbilical cord for promoting nerve regeneration in an axonotmesis rat model. *Neural Regen Res* 2012;**7**:2247–58.
23. Pereira T, Armada-da Silva PA, Amorim I *et al*. Effects of human mesenchymal stem cells isolated from wharton's jelly of the umbilical cord and conditioned media on skeletal muscle regeneration using a myectomy model. *Stem Cells Int* 2014;**2014**:1.
24. Pereira T, Gartner A, Amorim I *et al*. Promoting nerve regeneration in a neurotmesis rat model using poly(DL-lactide-epsilon-caprolactone) membranes and mesenchymal stem cells from the Wharton's jelly: in vitro and in vivo analysis. *Biomed Res Int* 2014;**2014**:1.
25. Pereira T, Ivanova G, Caseiro AR *et al*. MSCs conditioned media and umbilical cord blood plasma metabolomics and composition. *PLoS One* 2014;**9**:e113769.
26. Ribeiro J, Gartner A, Pereira T *et al*. Perspectives of employing mesenchymal stem cells from the Wharton's jelly of the umbilical cord for peripheral nerve repair. *Int Rev Neurobiol* 2013;**108**:79–120.
27. Ribeiro J, Pereira T, Amorim I *et al*. Cell therapy with human MSCs isolated from the umbilical cord Wharton jelly associated to a PVA membrane in the treatment of chronic skin wounds. *Int J Med Sci* 2014;**11**: 979–87.
28. Ribeiro J, Pereira T, Caseiro AR *et al*. Evaluation of biodegradable electric conductive tube-guides and mesenchymal stem cells. *World J Stem Cells* 2015;**7**:956–75.
29. Silva DM, Caseiro AR, Amorim I *et al*. Inflammatory response to dextrin-based hydrogel associated with human mesenchymal stem cells, urinary bladder matrix and Bonelike(R) granules in rat subcutaneous implants. *Biomed Mater* 2016;**11**:065004.
30. Caseiro A, Ivanova G, Pedrosa S *et al*. Human umbilical cord blood plasma as an alternative to animal sera for mesenchymal stromal cells in vitro expansion – a multicomponent metabolomic analysis. *PLoS One* 2018;**13**:e0203936. doi: 10.1371/journal.pone.0203936.
31. Kyllönen L, Haimi S, Mannerström B *et al*. Effects of different serum conditions on osteogenic differentiation of human adipose stem cells in vitro. *Stem Cell Res Ther* 2013;**4**:17.
32. Atayde LM, Cortez PP, Pereira T *et al*. A new sheep model with automatized analysis of biomaterial-induced bone tissue regeneration. *J Mater Sci Mater Med* 2014;**25**:1885–901.
33. Le Blanc K, Tammik C, Rosendahl K *et al*. HLA expression and immunologic propertiesof differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003;**31**:890–6.
34. Nuss K, von Rechenberg B. Biocompatibility issues with modern implants in bone - a review for clinical orthopedics. *Open Orthop J* 2008;**2**:66–78.
35. Caseiro AR, Alvites R, Pedrosa SS *et al*. The potential clinical application of mesenchymal stem cells from the dental pulp (DPSCs) for bone regeneration. *Frontiers in Stem Cell and Regenerative Medicine Research* 2017; **6**:3–52.
36. Laino G, Graziano A, d'Aquino R *et al*. An approachable human adult stem cell source for hard-tissue engineering. *J Cell Physiol* 2006;**206**:693–701.
37. Papaccio G, Graziano A, d'Aquino R *et al*. Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. *J Cell Physiol* 2006;**208**:319–25.
38. Kerkis I, Ambrosio CE, Kerkis A *et al*. Early transplantation of human immature dental pulp stem cells from baby teeth to golden retriever muscular dystrophy (GRMD) dogs: local or systemic? *J Transl Med* 2008;**6**:35.
39. Gronthos S, Mankani M, Brahim J *et al*. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci USA* 2000;**97**: 13625–30.
40. Bakopoulou A, Leyhausen G, Volk J *et al*. Comparative analysis of in vitro osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP). *Arch Oral Biol* 2011;**56**:709–21.
41. Nakamura H, Saruwatari L, Aita H *et al*. Molecular and biomechanical characterization of mineralized tissue by dental pulp cells on titanium. *J Dent Res* 2005;**84**:515–20.
42. Graziano A, d'Aquino R, Angelis MGCD *et al*. Scaffold's surface geometry significantly affects human stem cell bone tissue engineering. *J Cell Physiol* 2008;**214**:166–72.
43. Maiti SK, Shivakumar MU, Mohan D *et al*. Mesenchymal stem cells of different origin-seeded bioceramic construct in regeneration of bone defect in rabbit. *Tissue Eng Regener Med* 2018;**15**:477–92.

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Supplementary Information

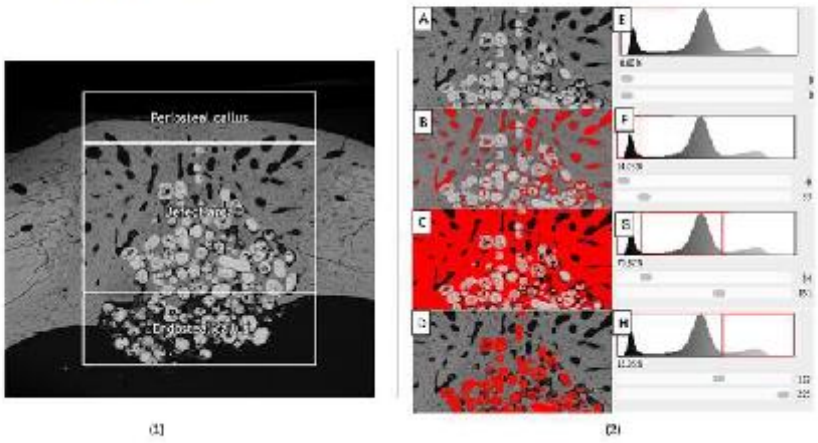


Figure S1: SEM image of bone defect divided into three different fractions: periosteal callus, defect area and endosteal callus (Amplification: 30x). Threshold analysis of a SEM image of bone tissue where Bonelike® **(A - D)** was implanted. The image segmentation was performed using histograms (E - H). A – Original image; B – With bone gaps highlighted (red); C – With bone highlighted (red); D – With Biomaterial highlighted (red).

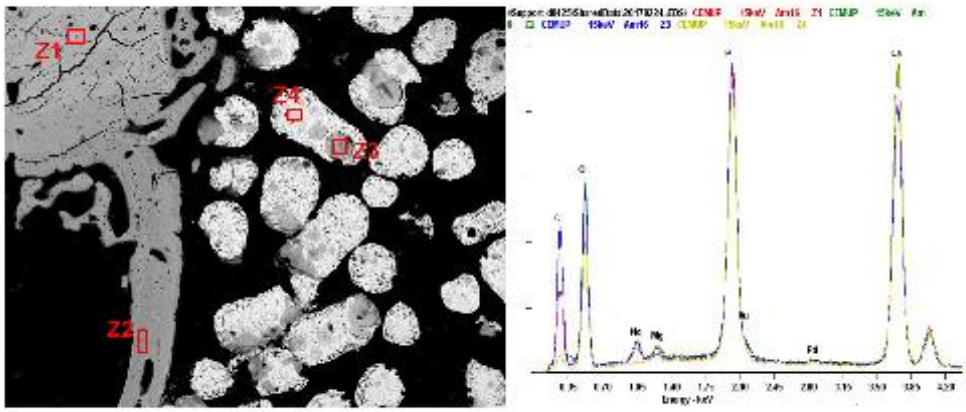


Figure S2: Image SEM of bone defect with respective EDS at 30 days of implantation. Red - Old bone (Z1); Dark green – Neo-formed bone (Z2); Blue - material incorporated inside Bonelike® (Z3); Yellow - Bonelike® (Z4) (Amplification: 100x).

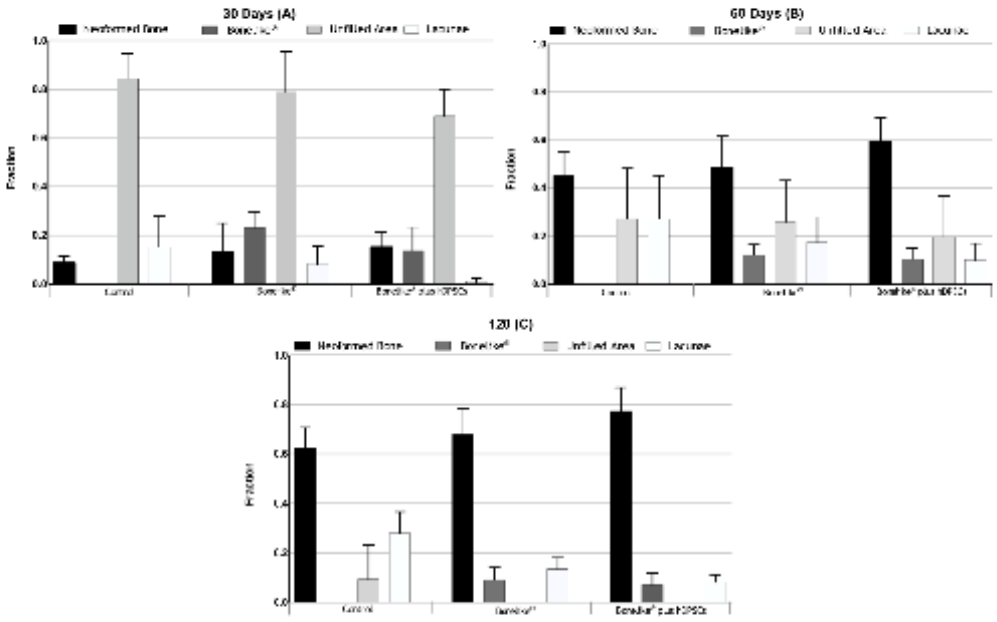


Figure S3: Histomorphometric analysis of bone defects with the Mean ± Standard deviation of the neo-formed bone, Bonelike®, unfilled area and lacunae fractions, at defect area, in 30 days (graphic A), 60 days (graphic B) and 120 days (graphic C).

Table S1: Statistical significance of histomorphometric results at 30 days of implantation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to 0.01≤P<0.05; 0.001≤P< 0.01; 0.0001≤P<0.001 and P<0.0001, respectively; ns, not significant.

			30 days											
			Control				*Bonelike®*				*Bonelike® plus hDPSCs*			
Histomorphometric Statistical significance			Neoformed Bone	Bonelike®	Unfilled Area	Lacunae	Neoformed Bone	Bonelike®	Unfilled Area	Lacunae	Neoformed Bone	Bonelike®	Unfilled Area	Lacunae
30 days	*Control*	Neoformed Bone		ns	****	ns	ns	ns	****	ns	ns	ns	****	ns
		Bonelike®			****	ns	ns	***	****	ns	**	*	****	ns
		Unfilled Area				****	****	****	ns	****	****	****	**	****
		Lacunae					ns	ns	****	ns	ns	ns	****	*
	Bonelike®	Neoformed Bone						ns	****	ns	ns	ns	****	ns
		Bonelike®							****	ns	ns	ns	****	****
		Unfilled Area								****	****	****	ns	****
		Lacunae									ns	ns	****	ns
	Bonelike® plus hDPSCs	Neoformed Bone										ns	****	****
		Bonelike®											****	****
		Unfilled Area												****
		Lacunae												

Table S2: Statistical significance of histomorphometric results at 60 days of implantation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to 0.01≤P<0.05; 0.001≤P< 0.01; 0.0001≤P<0.001 and P<0.0001, respectively; ns, not significant.

			60 days											
			Control				*Bonelike®*				*Bonelike® plus hDPSCs*			
		Histomorphometric Statistical significance	Neoformed Bone	Bonelike®	Unfilled Area	Lacunae	Neoformed Bone	Bonelike®	Unfilled Area	Lacunae	Neoformed Bone	Bonelike®	Unfilled Area	Lacunae
60 days	*Control*	Neoformed Bone		****	ns	ns	ns	****	*	***	ns	****	****	****
		Bonelike®			***	***	****	ns	**	ns	****	ns	**	ns
		Unfilled Area				ns	*	ns	ns	ns	****	*	ns	*
		Lacunae					*	ns	ns	ns	****	*	ns	*
	Bonelike®	Neoformed Bone						****	**	****	ns	****	****	****
		Bonelike®							ns	ns	****	ns	ns	ns
		Unfilled Area								ns	****	ns	ns	*
		Lacunae									****	ns	ns	ns
	Bonelike® plus hDPSCs	Neoformed Bone										****	****	****
		Bonelike®											ns	ns
		Unfilled Area												ns
		Lacunae												

Table S3: Statistical significance of histomorphometric results at 120 days of implantation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to 0.01≤P<0.05; 0.001≤P< 0.01; 0.0001≤P<0.001 and P<0.0001, respectively; ns, not significant.

			120 days											
			Control				*Bonelike®*				*Bonelike® plus hDPSCs*			
Histomorphometric Statistical significance			Neoformed Bone	Bonelike®	Unfilled Area	Lacunae	Neoformed Bone	Bonelike®	Unfilled Area	Lacunae	Neoformed Bone	Bonelike®	Unfilled Area	Lacunae
120 days	*Control*	Neoformed Bone		****	****	****	ns	****	****	****	****	****	****	****
		Bonelike®			ns	****	****	ns	ns	**	****	ns	ns	ns
		Unfilled Area				****	****	ns	ns	ns	****	ns	*	ns
		Lacunae					****	****	****	***	****	****	****	****
	Bonelike®	Neoformed Bone						****	****	****	*	****	****	****
		Bonelike®							ns	ns	****	ns	*	ns
		Unfilled Area								**	****	ns	ns	ns
		Lacunae									****	ns	****	ns
	Bonelike® plus hDPSCs	Neoformed Bone										****	****	****
		Bonelike®											*	ns
		Unfilled Area												**
		Lacunae												

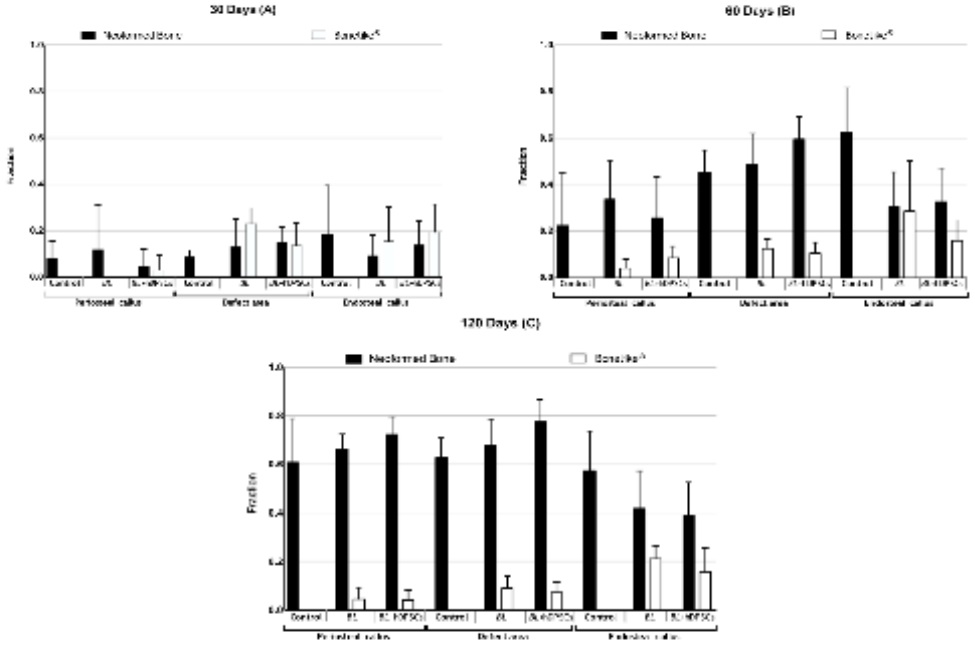


Figure S4: Histomorphometric analysis of bone defects with the Mean ± Standard deviation of the Neo-formed Bone and Bonelike® with group Control, Bonelike® and Bonelike® *plus* hDPSCs, divided into three different fractions: periosteal callus, defect area and endosteal callus, in the respective implantation times 30 days (graphic A), 60 days (graphic B) and 120 days (graphic C).

Table S4: Statistical significance of histomorphometric results at 30 days of implantation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to 0.01≤P<0.05; 0.001≤P< 0.01; 0.0001≤P<0.001 and P<0.0001, respectively; ns, not significant.

Histomorphometric Statistical significance				*30 days*																			
				Periosteal callus						*Defect area*						*Endosteal callus*							
				Control		*Bonelike®*		*Bonelike® plus hDPSCs*		*Control*		*Bonelike®*		*Bonelike® plus hDPSCs*		*Control*		*Bonelike®*		*Bonelike® plus hDPSCs*			
				Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®		
30 days	*Periosteal callus*	*Control*	Neoformed Bone		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	
			Bonelike®			ns	ns	ns	ns	ns	ns	ns	**	*	ns	ns	ns	ns	ns	ns	ns	**	
		Bonelike®	Neoformed Bone				ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	
			Bonelike®					ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	**	
		Bonelike® plus hDPSCs	Neoformed Bone						ns	ns	ns	ns	*	*	ns	ns	ns	ns	ns	ns	ns	****	
			Bonelike®							ns	ns	ns	**	**	**	*	ns	ns	ns	**	****		
	Defect area	*Control*	Neoformed Bone								ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns		
			Bonelike®									ns	**	ns	ns	ns	ns	ns	ns	*	**		
		Bonelike®	Neoformed Bone										ns	ns	ns	ns	ns	ns	ns	ns	ns		
			Bonelike®											ns	ns	ns	**	ns	ns	ns	ns		
		Bonelike® plus hDPSCs	Neoformed Bone												ns	ns	*	ns	ns	ns	ns		
			Bonelike®													ns	ns	ns	ns	ns	ns		
	Endosteal callus	*Control*	Neoformed Bone														ns	ns	ns	ns	ns		
			Bonelike®															ns	ns	ns	**		
		Bonelike®	Neoformed Bone																ns	ns	ns		
			Bonelike®																	ns	ns		
		Bonelike® plus hDPSCs	Neoformed Bone																		ns		
			Bonelike®																				

Table S5: Statistical significance of histomorphometric results at 60 days of implantation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant.

Histomorphometric Statistical significance				*60 days*																	
				Periosteal callus						*Defect area*						*Endosteal callus*					
				Control		*Bonelike®*		*Bonelike® plus hDPSCs*		*Control*		*Bonelike®*		*Bonelike® plus hDPSCs*		*Control*		*Bonelike®*		*Bonelike® plus hDPSCs*	
				Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®
60 days	*Periosteal callus*	*Control*	Neoformed Bone		*	ns	ns	ns	ns	*	*	**	ns	****	ns	****	*	ns	ns	ns	ns
			Bonelike®			****	ns	***	ns	****	ns	****	ns	****	ns	****	ns	****	***	****	ns
		Bonelike®	Neoformed Bone				****	ns	****	ns	****	ns	*	****	***	***	****	ns	ns	ns	*
			Bonelike®					**	ns	****	ns	****	ns	****	ns	****	ns	**	**	****	ns
		Bonelike® plus hDPSCs	Neoformed Bone						***	*	***	***	ns	****	**	****	***	ns	ns	ns	ns
			Bonelike®							****	ns	****	ns	****	ns	****	ns	**	**	****	ns
	Defect area	*Control*	Neoformed Bone								****	ns	****	ns	****	ns	****	ns	ns	ns	****
			Bonelike®									****	ns	****	ns	****	ns	****	***	****	ns
		Bonelike®	Neoformed Bone										****	ns	****	ns	****	ns	ns	ns	****
			Bonelike®											ns	****	ns	****	ns	ns	ns	****
		Bonelike® plus hDPSCs	Neoformed Bone												****	ns	****	****	****	****	****
			Bonelike®													****	ns	**	*	****	ns
	Endosteal callus	*Control*	Neoformed Bone														****	****	****	****	****
			Bonelike®															****	***	****	ns
		Bonelike®	Neoformed Bone																ns	ns	ns
			Bonelike®																	ns	ns
		Bonelike® plus hDPSCs	Neoformed Bone																		**
			Bonelike®																		

Table S6: Statistical significance of histomorphometric results at 120 days of implantation. Significance of the results is indicated according to P values with one, two, three or four of the symbols (*) corresponding to $0.01 \leq P < 0.05$; $0.001 \leq P < 0.01$; $0.0001 \leq P < 0.001$ and $P < 0.0001$, respectively; ns, not significant.

				120 days																	
				Periosteal callus						Defect area						Endosteal callus					
				Control		Bonelike®		Bonelike® plus hDPSCs		Control		Bonelike®		Bonelike® plus hDPSCs		Control		Bonelike®		Bonelike® plus hDPSCs	
Histomorphometric Statistical significance				Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®	Neoformed Bone	Bonelike®
120 days	Periosteal callus	Control	Neoformed Bone		****	ns	****	ns	****	ns	****	ns	****	**	****	ns	****	**	****	****	****
			Bonelike®			****	ns	****	ns	****	ns	****	ns	****	ns	****	ns	****	***	****	**
		Bonelike®	Neoformed Bone				****	ns	****	ns	****	ns	****	ns	****	****	****	****	****	****	ns
			Bonelike®					****	ns	****	ns	****	ns	****	ns	****	****	*	****	ns	****
		Bonelike® plus hDPSCs	Neoformed Bone						****	ns	****	ns	****	ns	****	**	****	****	****	****	****
			Bonelike®							****	ns	****	ns	****	ns	****	ns	****	***	****	**
	Defect area	Control	Neoformed Bone								****	ns	ns	ns	ns	****	**	****	ns	****	ns
			Bonelike®									****	*	****	ns	****	***	****	****	****	****
		Bonelike®	Neoformed Bone										ns	****	ns	****	ns	****	***	****	**
			Bonelike®											****	ns	****	ns	****	ns	****	ns
		Bonelike® plus hDPSCs	Neoformed Bone												****	****	****	****	****	****	****
			Bonelike®													****	ns	****	*	****	ns
	Endosteal callus	Control	Neoformed Bone														****	ns	****	***	****
			Bonelike®															****	***	****	**
		Bonelike®	Neoformed Bone																***	ns	****
			Bonelike®																	***	ns
		Bonelike® plus hDPSCs	Neoformed Bone																		****
			Bonelike®																		

Chapter 8 – Clinical Cases – Bonelike® applications

Introduction

Animals have been part of human households since humans started living in villages, approximately 12,000 years ago. Almost all cultures have maintained a commitment to caring and protecting animals kept solely for companionship [330].

It is difficult to know how many pets exactly are in the world because the answer is frequently based on owned or registered dogs, but according to the American Veterinary Medicine Association and to the American Pet Products Association's there are 60 millions of companion dogs in the world and 80 million just in the USA [331, 332].

Nonetheless, the total number of dogs on the planet is approximately 525 million. China is home to about 110 million dogs, Africa about 78 million owned dogs (another 70 million unowned dogs), France about 8.8 million and Japan, a country with most of their dogs registered, is 9.5 million [331].

The human-animal binomial and the quality of Veterinary Medicine services leads to an increase in the elderly dog population that will show increasing prevalence of age-associated diseases in need of treatment. This can lead to a complete paradigm in translational research where instead of inducing diseases in laboratory animals, dog patients in need of treatment are recruited and engaged citizens become involved in the research. Also aims at improving the companion dog welfare provide complex inputs for development of human therapies [332].

In Veterinary Medicine, a variety of biological and mechanical factors associated with particularities of each animal and injury, can contribute to the occurrence of nonunion and often the cause of nonunion may be multifactorial. A substantial proportion of fractures can present with mal unions and/or nonunion, and the management of nonunion continues to present a challenge for orthopedic surgeons. Successful management often requires assessment and treatment of more than one of these factors. Most common factors that may contribute to mal-union and/or nonunion include infection, impaired biology, and metabolic disorders. Over the last

decade there has been a substantial increase in the number of new bone graft substitutes available to the orthopedic surgeon to assist bone healing [98].

In addition, new and evolving strategies for diagnosing the cause and effectively treating mal unions and/or nonunion include the diagnosis of infection, metabolic workup, bone grafting, cell-based therapies, and biological adjuvants are reviewed and discussed. There is a large range of bone repair strategies that are adapted to different clinical needs [39, 333, 334].

The use of bone grafts is recommended in numerous orthopedic surgeries. The autograft is considered to be the technique of choice in bone fusions, for small gaps and in critical defects given the properties of osteoconduction, osteogenesis and osteoinduction. Regardless of its unique characteristics, there are an increasing substitution with synthetic bone due to the high morbidity of the harvest and limitations of the volume of autografts. Also the well-known disadvantages of allografts, such as potential risk for transmission of infectious diseases and the induction of adverse immune reactions, have driven the development of synthetic bone grafts for bone regeneration both in human and in veterinary medicine. For those reasons in recent decades there has been an enlargement of the need to use bone grafts [193].

Bonelike® in the form of spherical granules (250-500 μm) has been tested in several clinical situations and has proven its excellent biocompatibility and bioactivity [79]. This is evidenced by clinical trials in animals [82, 133, 194, 335-338] and humans [79, 144, 339]. This synthetic bone substitute, produced based on a mixture of HA ($Ca_{10}(PO_4)_6(OH)_2$) and TCP ($Ca_3(PO_4)_2$), can be used in clinical situations of bone regeneration has already been tested in filling bone defects after the removal of cysts, in implant after surgical removal of impacted teeth, the bone filling bags of two or more walls, as well as junctions or trifurcations teeth, in elevation of the maxillary sinus bone and orthopedic applications [113, 137].

Several studies using Bonelike® implants concluded that this type of graft was highly osteoconductive and that it was a material with good potential to be used in clinical cases of reconstructive surgery. The bone was formed quickly around of Bonelike® implants, concluding that it was more osteoconductive comparing to HA [133].

Furthermore, the inclusion of the glass part during the production process allows the incorporation of different ions in the HA matrix, like fluorine, magnesium and sodium, approaching the final composition of Bonelike®, the composition of the mineral phase of bone [200].

In many clinical situations, particularly in the more severe bone disease, as with patients of advanced age and / or suffering from osteoporosis, it becomes absolutely necessary to adopt new methods of preparation and application of bone substitutes. Accordingly, there was a need to develop and optimize bone substitutes, based vehicles suitable for regeneration of bone tissue polymeric stabilizers, facilitating the simultaneous application of therapeutic molecules or microelements favor such regeneration. Thus, allow the slow release of these molecules, favoring the maintenance of physiological concentrations at the site of its application [198].

Bonelike® has a highly bioactive behavior, demonstrated in several *in vitro* and *in vivo* studies [133, 336] together with some clinical trials [82, 116]. Granular forms of this biomaterial were applied in orthopaedics and dental applications demonstrating the benefits of its properties for bone regeneration. However, these studies also highlighted the need to develop methods towards a fast consolidation of Bonelike® when applied in unstable sites, along with an improvement of its handling properties and injectability if used in minimally invasive surgical techniques. The possibility of further improving its *in vivo* behavior was also considered. The bone substitute Bonelike® has been tested in numerous clinical situations (using animals and humans) and has proven its excellent biocompatibility and bioactivity [82, 116].

Extensive bone loss from trauma, tumour resection, or infection is associated with impaired healing, the adequate treatment of which has remained a longstanding clinical problem. Bone defects above a certain critical size, typically 3 cm or larger in humans, exceed the natural regenerative capacity of bone and frequently result in non-union or delayed healing even with surgical intervention [7].

However, repairing a large bone defect using bone autografts becomes more impractical with increasing defect size, due to the large volume of bone that needs to be harvested from the donor site, which is further complicated by the high resorption rates of autologous bone after implantation. Driven by the limited supply of bone

autografts and their increased risk of complications due to second-site surgery, as well as the inability of bone allografts to achieve the same healing outcomes as autografts due to reduced bioactivity, a number of bone graft substitutes have faced the market in the past 30 years with many more still in development [83].

In many clinical situations, particularly in the more severe bone disease, as with patients of advanced age and/or suffering from osteoporosis, it becomes absolutely necessary to adopt new methods of preparation and application of bone substitutes. Accordingly, there is a need to develop and optimize bone substitutes suitable for regeneration of bone tissue polymeric stabilizers, facilitating the simultaneous application of therapeutic molecules or microelements that favour such regeneration [198].

Block autograft is considered a “gold standard” in cases of severe bone atrophy, owing to high osteoconductive and osteogenic potentials [340], without the risks of immune response and disease transmission [341]. However, limitations exist with block autograft, such as increased operation time, donor site morbidity and a limited amount of intra-orally harvested bone [342]. With recent developments, xenograft or synthetic bone grafts in block form represent an alternative approach to using autogenous bone and thus eliminate these drawbacks [343].

The regeneration and establishment of osseointegration within oral peri-implant bone defects remains a clinical challenge. Bone tissue engineering is emerging as a promising alternative to autogenous and/or biomaterial-based bone grafting. Osseointegrated implants are an integral part of modern reconstructive dentistry, and are associated with favorable long-term therapeutic outcomes . Adequate bone volume allowing placement of implants in functionally and/or esthetically optimal positions is an important prerequisite for success. However, insufficient alveolar bone volume, as a result of periodontal disease, trauma, and/or resorption atrophy, often presents a clinical challenge for implant installation. Further, implant placement in deficient bone or in extraction sockets with compromised bone walls may result in horizontal and/or vertical defects exposing the implant body and potentially compromising short- and long-term outcomes [77].

The possibility of further improvements *in vivo* behaviour was considered and in this part of my Doctoral thesis, I present several clinical cases that were developed at HVUC and UP-Vet.

This pre-clinical trial intends to contribute to the development of new strategies in the context of bone regeneration surgery, targeting a rapid restoration of the skeletal integrity and reduction of the associated morbidity where biomaterials such as ceramics play an important role. In the present study, we tested synthetic bone graft Bonelike® in the form of spherical granules (250-500 μm) on non critical bone defects during small animal clinical cases. All animal´s tutors signed a consent informed sheet form before surgeries (see **Attachments**).

Chapter 9 – Application of Bonelike® as synthetic bone graft in orthopaedic and oral surgery in veterinary clinical cases

During my PhD research I also performed surgery in small animal clinical cases, particularly in orthopaedic and stomatology clinical fields, using a synthetic bone graft substitute composed of a modified HA matrix in the form of spherical granules with 250-500µm of diameter. Bonelike® is a biomaterial that overcomes some of the main disadvantages of traditional HA and the disadvantages documented for the autografts. Bonelike® presents a better osteoinductive and osteoconductive proprieties and I was the first to use it in the dogs and cats.



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SHORT REPORT Open Access

Application of Bonelike® as synthetic bone graft in orthopaedic and oral surgery in veterinary clinical cases

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Abstract

Autologous bone remains the gold standard grafting substrate for bone fusions used for small gaps and critical defects. However, significant morbidity is associated with the harvesting of autologous bone grafts and, for that reason, alternative bone graft substitutes have been developed. In the present case series, a glass-reinforced hydroxyapatite synthetic bone substitute, with osteoinductive and osteoconductive proprieties, was applied. This synthetic bone substitute comprises the incorporation of P_2O_5-CaO glass-based system within a hydroxyapatite matrix, moulded into spherical pellets with 250-500 μm of diameter. A total of 14 veterinary clinical cases of appendicular bone defects and maxillary / mandibular bone defects are described. In all clinical cases, the synthetic bone substitute was used to fill bone defects, enhancing bone regeneration and complementing the recommended surgical techniques. Results demonstrated that it is an appropriate synthetic bone graft available to be used in veterinary patients. It functioned as a space filler in association with standard orthopaedic and odontological procedures of stabilization, promoting a faster bone fusion without any local or systemic adverse reactions. This procedure improves the animals' quality of life, decreasing pain and post-operative recovery period, as well as increasing bone stability improving positive clinical outcomes.

Keywords: Biomaterial, Bonelike®, Synthetic bone graft, Bone regeneration, Case series, Veterinary, Canine, Feline, Orthopaedics, And odontology

Introduction

With increasing average life expectancy [1] the number of degenerative diseases, osteogenic disorders and bone fractures has scaled in recent decades [2]. Bone defects due to trauma, pathological and physiological bone resorption represent a major challenge and are a global health problem [3], in both human and domestic animal individuals. The traditional methods for repairing bone defects (such as autografts, allografts and xenografts) bare noteworthy disadvantages, thus limiting their clinical application [4, 5]. Some of these bone grafts can be resorbed in a time controlled way in order to allow the correct process of natural re-construction of the involved bone tissue to occur and restore skeletal integrity [3]. Large bone defect repair is a difficult problem to be solved urgently in the orthopaedic field and the application of bone repair materials appears as a viable alternative [6]. Recent development of calcium phosphate ceramics and other related biomaterials for bone graft provide improved control of the process of resorption and the ability for new bone to form. Glass-based ceramics, such as bioactive glasses, and calcium phosphates [calcium hydroxyapatite; tricalcium phosphate (TCP) and biphasic calcium phosphate] are popular materials for mineralised tissue regeneration [7]. Santos et al.

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demonstrated that hydroxyapatite bioactivity is improved through the combination with a P_2O_5-CaO-Na_2O glass system (resulting biomaterial patented as Bonelike®) [8]. Bonelike® is composed by a modified hydroxyapatite (HA) matrix, with α- and β-tricalcium phosphate secondary phases, that counterpart HA's brittle and essentially non-degradable character, improving the bioresorption of the modified system [9]. The structure of Bonelike® presents a computer controlled 3D architecture, and a complex composition that mimics the mineral composition of natural bone [10, 11]. Further, Bonelike® bares enhanced bioactivity by reproducing the inorganic phase of HA in bone which contains several ionic substitutions which modulate its biological behavior. Additionally, the mechanical properties of the temporary graft are improved by the CaO-P_2O_5 based glasses [12, 13].

The main indications for Bonelike® applications include non-unions, delayed unions, *mal* unions, bone defects, bone cysts, tumours and arthrodesis [14], and its clinical application has been reported in craniofacial, oral/maxillofacial, orthopedic and dental surgery [15]. The clinical applications in maxillary bone defects indicated good bonding between newly formed bone and the Bonelike® granules [16].

In Veterinary Medicine, fresh cancellous bone graft remains the gold standard in veterinary orthopaedics for enhancing defect healing. Fortunately, the use of synthetic bone grafts has been increasing in the last two decades, and the application of β-TCP derivates, like Bonelike®, has already stepped out of preliminary pre-clinical assays to veterinary applications [17]. Due to the costs involved in the use of biomaterials, its use is still not as frequent as in Human Medicine. However, the evolution of social consciousness and attention to pets is reflecting in increased disposition of owners to invest in the treatment of their animals.

Several case reports detail on the application of bone substitutes in veterinary patients. Successful resolution of *pes varus* in two miniature Daschshunds has been reported, using a wedge of synthetic β-TCP to fill the gaps created by *tibial* corrective open osteotomies. According to Izamisawa et al [18], 2 months after the surgery the implanted edges were integrated with native bone. The bone plates and screws were removed after 4 months and, by then, the TCP wedges were completely resorbed, and the osteotomy bone remodelled. The use of TCP avoided the need of a second surgery to harvest autologous cancellous bone graft. The postoperative angles were corrected and maintained during the follow-up period. Morphologically, the body of the tibia in the affected hind limb nearly equalled the one found in healthy limbs [19]. In a similar approach, granules of β-TCP were mixed with fresh blood and used as void filler in subcritical-sized bone defects in long bones in 13 animals. All but one case achieved complete bone union, and radiographic bone ingrowth ranged from 75 to 100%. The study reported excellent clinical results confirming the biocompatibility and usefulness of β-TCP as a synthetic bone graft for moderate to large subcritical bone defects with initially expected good biological conditions such as blood supply and cellular activity [20]. Another case report refers to a distal radius atrophic non-union in a one-year old male Yorkshire Terrier, using a 3D-printed β-TCP scaffold with bone morphogenic proteins (rhBMP-2) (truScient®) to create a scaffold with the same shape as the defect. After the removal of the bone plate, the scaffold was no longer visible and complete corticalization of the regenerated bone area was observed on computerized tomography (CT) scan evaluation. The scaffold was deemed an excellent bone substitute due to good osteoinductive properties of rhBMP-2, complemented with good osteoconductive potential provided by the open-interconnected pores from the β-TCP scaffold [20].

Herein we report a case series of 14 appendicular bone defects and maxillary / mandibular bone defects in which the surgical approach included the use of Bonelike® as a bone substitute, aiming at the optimization of the selected intervention outcomes.

Materials and methods

Case selection and general procedures

The case series includes 14 orthopaedic clinical cases in small animals, ten canine and four feline patients, presenting moderate to severe non-critical bone defects.

Feline patients ranged from 4 to 16 years of age with an average age of 8 years, weighing 3 to 6 kg (average 4.25 kg), 2 females and 2 males. Canine patients were aged 1 to 14 years (average age of 7.7 years), weighing 2 to 30 kg (average 18.5 kg), 1 female and 9 males. Two of the canine cases were appendicular bone fractures and the remaining were maxillary and mandibular bone fractures. Legal tutors of all animals provided informed consent on the application of Bonelike® as part of the instituted therapy. Patients were selected strictly on the basis of their clinical needs and according to the following criteria, which included: patients of any age, gender or weight, patients without any systemic disease/ infection, and finally, all clinical situations in which the time of bone healing was expected to supplant the standard time. The exclusion criteria included the presence of systemic disease, infected cavities, acute infection at the local of bone defect, bone inflammatory diseases (particularly osteomyelitis), malignant tumours, severe renal dysfunctions, animals with increased anaesthesia risk and animals with non-controlled bone metabolism. Individual data of each clinical case is summarized in Table 1:

Table 1 Individual data of the animals included in the case series

Case n° #	Breed	Sex – Age – Body weight	Problem	Bone defect grading	Surgical procedure (reduction system)	Consolidation time of the lesion (weeks)	Functional Recovery	Skeletal system	Gender
1	**Spitz**	Male - 1y - 5 kg	Non-union defect of radius and ulna after two previous surgical failure	Large	Corrective osteotomy of radius with osteosynthesis plate in radius and another plate in ulna	12	Good	Appendicular bone defects	Canine
2	**Labrador retriever**	Male - 3y – 30 kg	Traumatic tibia fracture with a non-union defect after one previous surgical failure	Large	Tibia fracture repair with osteosynthesis plate	12	Good		
3	**Mixed breed**	Female - 8y - 25 kg	Traumatic mandibular fracture with a delay healing after one interdental wiring failure	Small	Mandibular reconstruction with osteosynthesis plate	8	Excellent	Maxillary and mandibular bone defects	
4	**Collie**	Male - 10y - 15 kg	Traumatic rostral mandibular fracture with loss of canine tooth	Medium	Mandibular reconstruction with external skeletal fixation	5	Excellent		
5	**Yorkshire Terrier**	Male - 4y - 2 kg	Periodontic-endodontic disease	Small	Reconstruction of alveolar cleft after a maxillary tooth extraction	4	Excellent		
6	**Mixed breed**	Male - 14y - 9 kg	Periodontic-endodontic disease	Small	Unilateral reconstruction of alveolar cleft after exodontic procedure	6	Excellent		
7	**Mixed breed**	Male – 15y -7 kg	Periodontal disease (oronasal communication)	Medium	Reconstruction of alveolar cleft after total premolar and molar tooth removal	12	Excellent		
8	**Pit Bull**	Male - 11y - 28 kg	Canine tooth fracture	Large	Extraction of one canine tooth	12	Excellent		
9	**Labrador retriever**	Male - 3y - 28 kg	Canine tooth fracture	Small	Extraction of one canine tooth	8	Good		
10	**Labrador retriever**	Male – 8y – 34 kg	Molar extraction	Small	Extraction of one molar tooth	8	Good		
11	*European Shortair*	Male - 7y - 4 kg	Periodontic-endodontic disease	Medium	Reconstruction of alveolar cleft after total premolar and molar tooth removal	12	Excellent		Feline
12	*European Shortair*	Female - 5y - 6 kg	Periodontitis (root reabsorption)	Small	Reconstruction of alveolar cleft after a maxillary tooth extraction	4	Excellent		
13	*European Shortair*	Male - 16y - 4 kg	Traumatic mandibular fracture	Small	Fracture repair with external fixation	12	Good		
14	*European Shortair*	Female - 4y - 3 kg	Traumatic mandibular fracture	Small	Fracture repair with external fixation	12	Good		

Bold data are canine patients. Italic data are feline patients

species, breed, sex, age, weight, diagnosis (motive for the surgery) and surgical procedure performed. Due to the disparity of animals' size and affected bones, defects were graded/ classified empirically from small to large according to width and length of the defects [rather than being attributed a metric classification].

All surgical procedures were performed at the Veterinary Hospital of the University of Coimbra (HVUC) and the Veterinary Hospital of ICBAS from the University of Porto (UP-Vet). The animals were maintained at the referred hospitals and were fed twice a day with appropriate diet.

Before surgery, preventive food and water withdrawal was instituted for 8 and 2 h respectively. Preoperatively, patients were administered non-steroid anti-inflammatory drugs and opioids. Propofol was administered intravenously as general anaesthetic induction (dose adjusted to desired effect), and adequate anaesthesia depth maintained using sevoflurane. The surgical sites were shaved as needed and then scrubbed with disinfection solution. All the surgical approaches were undertaken in accordance to Halsted's surgical principles.

In all small animal clinical cases included, Bonelike® was used to enhance bone healing, to fill critical defects and complement the recommended surgical procedures. In all cases, spherical granules of Bonelike® with a diameter between 250 and 500 μm were mixed with fresh autologous blood (Fig. 1). A single-ended curette helped to create the mixture and administer it to fill the bone defects.

After the required surgical intervention, tissue incisions were sutured in layers with absorbable Monosyn®. Postoperatively, opioids were administered to reduce pain for 2 days, anti-inflammatory drugs for 6 days, and beta-lactam antibiotics were administered for 8 days.

The follow-up consults included general physical examination, control X-rays and client-owned feedback in order to monitor the process. Radiographic monitoring was performed periodically and compared to preoperative records (kVp and mA settings individually adjusted to each case; files saved as DICOM). To evaluate functional recovery, a simple scale with three degrees was admitted: excellent, good and bad, indicating total, partial and no recovery of function, respectively.

Clinical cases description

In clinical case #1, one-year-old toy breed dog (Spitz), with 5 kg of bodyweight (b.w.) presented a fracture of the radius and ulna with a large loss of bone, after a low-level trauma event. The patient was treated with an initial external skeletal fixation (modified type 2b) and subsequent neutralization osteosynthesis plate fixation. However, both corrective techniques applied failed.

On the third admission for surgery, the patient presented a severe atrophic non-union, classified as a critical large bone defect that was not expected to heal without additional intervention or bandage. Surgery was performed, and the synthetic bone graft Bonelike® was used, due to the limited availability of autologous cancellous bone for grafting. For the purpose, Bonelike® granules were mixed with autologous cancellous bone graft and deposited to fill the 45 mm-defect on the length of the radius, and a neutralizing bridge plate was applied. A complementary corrective ulnar osteotomy was performed with an additional neutralizing plate in order to improve bone alignment. Radiographic pre- and post-surgical control was performed and it is represented in Fig. 2. The functional recovery was good after 12 weeks, and radiographic control indicated good mineralization of the defect area and visible lines of ossification.

In clinical case #2, a 3-year-old male Labrador Retriever, weighing approximately 30 kg b.w., presented a *tibial* fracture with large bone defect, after traumatic fracture. Surgical correction was attempted with internal fixation of the bone fragments with cerclage wiring, but bone fragment dislodgement resulted in failure of the surgical approach described. In a secondary surgical intervention, Bonelike® was applied on the *tibial*

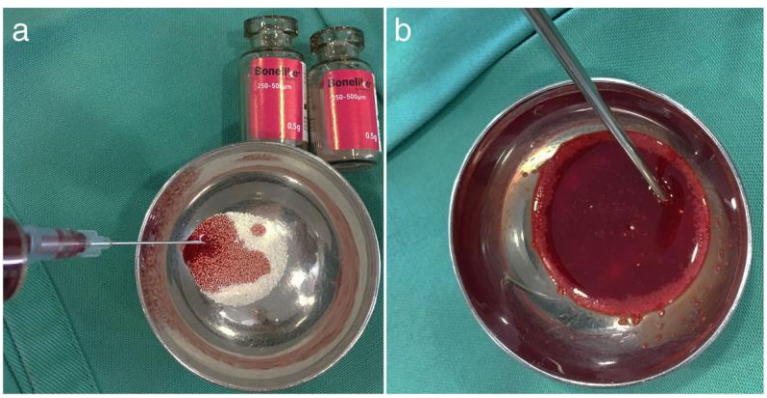


Fig. 1 Preparation of Bonelike® mixture for intraoperative application, through mixture with autologous blood (**a**); mixed clot prepared for application (**b**)

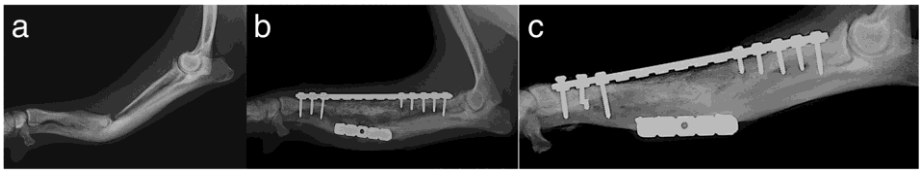


Fig. 2 Case #1 - Latero-lateral radiographic images of the front limb, presenting a critical radio-cubital bone defect. Preoperative non-union after two events of implant failure (**a**); Postoperative control 4 months after surgical correction using Bonelike®(**b**); Postoperative control 8 months after surgical correction using Bonelike®(**c**)

non-union, mixed with autologous cancellous bone, as well as crushed bone remnants and platelet rich plasma. Radiographic evaluation and clinical examination were performed in the follow-up periods (4, 8 and 12 months) to assess for the efficacy of the surgical treatments (Fig. 3). Clinical outcome for up to 12 months was graded as good and effective osteointegration was confirmed.

In the clinical case #3, an 8-year-old female mixed-breed, with 25 kg b.w., presented with a complex traumatic oblique mandibular fracture, which was primarily treated with fixation of a mandibular fracture using interdental wiring. However, the fracture borders overlapped delaying the healing process. At a second intervention, the fracture was reduced with a neutralizing osteosynthesis plate and the maxillary alveolar bone defect filled with Bonelike® spherical granules. Orthopedic jaw fixation was performed using plate and screws to avoid damage to the root structures or disrupt the dental vascular supply. After 8 weeks, radiographic control fracture consolidation (Fig. 4) and a very good recovery of masticatory function was described by the legal tutors.

In clinical case #4, an 8-year-old male Collie, weighing 15 kg b.w., presented a fracture of the left side rostral jaw, resulting in canine tooth avulsion (affecting the tooth roots, nerves, blood vessels and salivary ducts) which created a medium size bone defect, between the incisor and premolar teeth. The mandibular fracture was repaired with an external fixation system, and Bonelike® spherical granules were used to fill the mandibular space resulting from structure avulsion.

Follow-up consult after 5 weeks confirmed fracture consolidation and excellent ability to eat and drink was described by the legal tutors. The external fixation apparatus was removed uneventfully (Fig. 5).

In clinical case #5, a 4-year-old small male Yorkshire Terrier was presented for consult due to progressive weight loss, weighing 2 kg b.w. at the time of the veterinary clinical evaluation. Upon consult and examination, additional clinical signs were identified, such as anorexia, dysphagia, apparently unspecific pain and an external root resorption of the maxillary pre-molar tooth, possibly progressing into crown destruction (suggested by a pink hue commonly associated to changes in mineral consistency). Radiographic examination highlighted a lucency area at the root tip of the maxillary tooth. An exodontic treatment was recommended, but the fragility of this maxilla was a concern. To address this fragility and to avoid spontaneous fracture, Bonelike® was applied after the extraction, to fill the defect and restore the maxillary bone integrity. After 4 weeks, the oral / gingival mucosa was healed and radiographic evaluation revealed complete and homogenous mineralization of the maxillary bone. No adverse reactions were detected and the patient regained appetite and normal food intake, demonstrating an excellent functional recovery (Fig. 6).

In clinical case #6, a 14-year-old mixed-breed presented with severe periodontic and endodontic disease of the

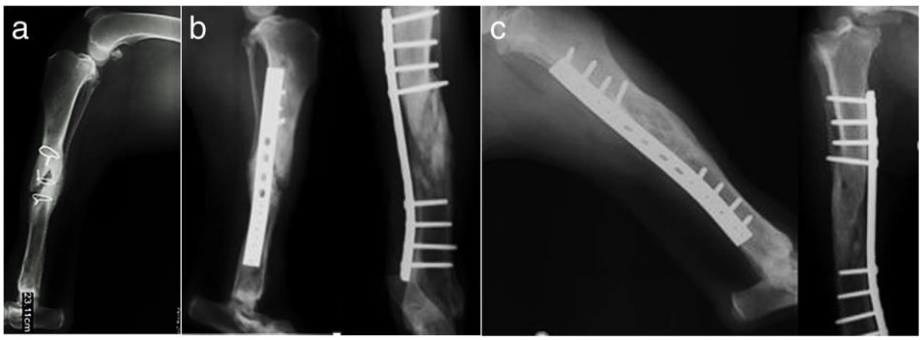


Fig. 3 Case #2 – Latero-lateral and craniocaudal radiographic images of the hind limb, presenting a critical *tibial* bone defect. Preoperative non-union after internal fixation failure (**a**); Immediate postoperative control after surgical correction using Bonelike®(**b**); Postoperative control 4 months after surgical correction using Bonelike®(**c**)

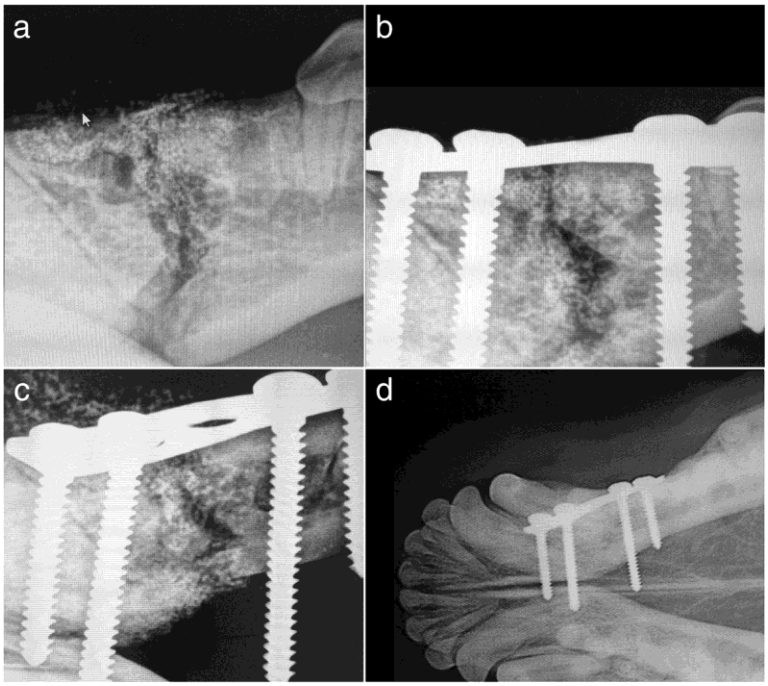


Fig. 4 Case #3 - Radiographic study of the mandibula presenting an oblique fracture and absence of adjacent teeth (**a**); Immediate postoperative control after surgical reduction by plate fixation and Bonelike® filling of the mandibular alveolar bone (**b**, **c**); Postoperative control 8 weeks after surgical correction using Bonelike®(**d**)

maxillary molar teeth. Radiographic examination revealed severe periodontal bone loss, with profound periapical lucency, indicative of endodontic disease and periodontitis. Total extraction was carried out and small alveolar bone spaces were asymmetrically created on the maxillary bones. Application of spherical Bonelike® was performed exclusively on the left maxilla, where bone compromise was more impressive, to avoid future nasal fistula, and the right maxilla was kept unfiled. No adverse reactions were observed, and a smooth recovery was reported by the legal tutor. At 6 weeks post-intervention, the clinical examination revealed great improvement of buccal health and increased bodyweight. Moreover, a mineralized consolidation of the left grafted area where it was applied the Bonelike®, was confirmed through radiographic analysis (Fig. 7), while minor radiopacity was observed in the right side.

In clinical case #7, a 15-year-old mixed-breed canine was admitted with severe periodontal disease, in a similar presentation to Case #6. The X-rays revealed a wide periodontal ligament space and a periapical lucency with teeth root resorption on both sides of the maxilla. The teeth that presented crown resorption had a pink hue due to the changes in the mineral composition of the crown. Extraction of all teeth caudal to the 1st maxillary premolar was advised. After the extraction, the alveolar bone defect was filled with Bonelike®, and a mucosal flap was sutured over the bone with no tension, to occlude a patent oronasal fistula (Fig. 8). Radiographic evaluation

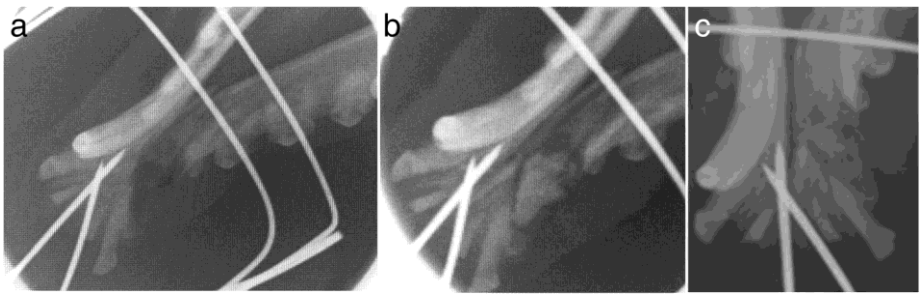


Fig. 5 Case #4 - Radiographic study of the mandibula presenting a mandibular fracture and absence of adjacent teeth (**a**); Immediate postoperative control after external fixation and Bonelike® filling of the alveolar bone defect (**b**); Postoperative control 5 weeks after surgical correction using Bonelike® (**c**)



Fig. 6 Case #5 - Radiographic study of the maxilla evidencing the alveolar defect resulting from exodontia of the 1st pre-molar tooth before (**a**) and after filling on the same with Bonelike® (**b**)

and clinical examination were performed 12 weeks after surgery to assess the efficacy of the surgical procedures. Adequate mineralization was observed, suggesting Bonelike® integration with the surrounding alveolar bone.

In clinical case #8, an 11-year-old male Pit Bull presented with a very common condition: a canine tooth crown fracture with no pulp exposure, and with periapical lucency surrounding the root upon radiographic assessment. Root canal therapy and crow reconstruction or extraction were the advised treatment options, as the maintenance of the damaged tooth would allow the bone destruction to progress. Pain was reported by the legal tutor as the main concern. Exodontia was selected as the treatment procedure, and once attained complete root exposition, the tooth, was removed. After the complete removal of the debris, the remaining defect was firmly packed with Bonelike® (mixed with autologous blood) to completely fill the cavity and cortical bone contour. Then a mucosal flap closure was performed, to prevent oronasal fistula formation. Care was taken to ensure sufficient mobility of the mucosal flap to cover the granules (Fig. 9). After 12 weeks the defect was completely mineralized, and the animal was eating well and without any concerns from the tutors.

In clinical case #9, a 3-year-old male Labrador Retriever presented with a similar defect under the maxillary mucosa of the canine tooth. Tooth extraction was performed and resulting bone cavity was filled with Bonelike®. Primary closure of the mucogingival flap was performed over the bone substitute filled defect. Immediate postoperative radiographic control indicated incomplete filling of the alveolar cavity with Bonelike® (Fig. 10). After 8 weeks the patient had an unremarkable examination of the mouth, showing complete recovery and bone regeneration of the maxillary defect.

In clinical case #10, a 8-year-old Labrador Retriever presented with a missing mandibular molar. Surgical exploratory surgery was undertaken, and the bone defect was filled with Bonelike® granules in order to promote the bone regeneration of the alveolar defect.

Recovery was positive and, 8 weeks after surgical approach, good mineralization was evident. Radiographic imaging as well as animal's behaviour and examination were unremarkable, confirming complete recovery and regeneration (Fig. 11).

In clinical case #11, a 7-year-old neutered male feline presented with periodontic-endodontic disease with associated stomatitis-gingivitis complex. After the extraction of molar and premolar teeth, spherical Bonelike® granules were applied in both maxillary alveolar defects. After 12 weeks, a good osteointegration was confirmed radiographically and no soft tissue nor systemic adverse reactions were detected (Fig. 12). Owners reported no pain, no swelling, no halitosis and overall a better quality of life.

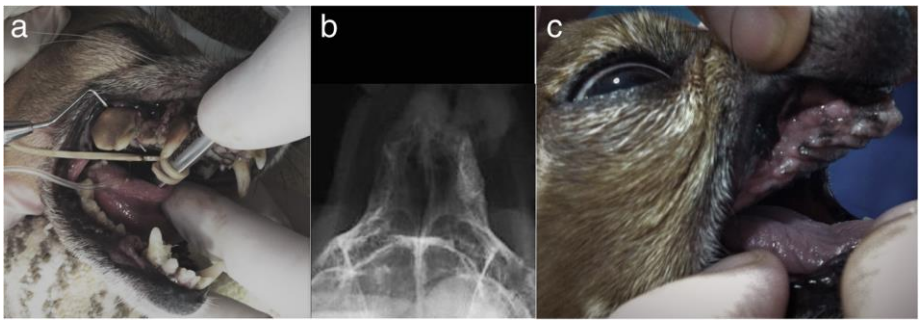


Fig. 7 Case #6 – Visual examination of the oral cavity affected by severe periodontal disease (**a**); Postoperative control 6 weeks after exodontic procedure and filling of the left maxillary cavity using Bonelike® (right side of the image) (**b**); Visual examination of the oral cavity 6 weeks after exodontic procedure and filling of the left maxillary cavity using Bonelike®(**c**)

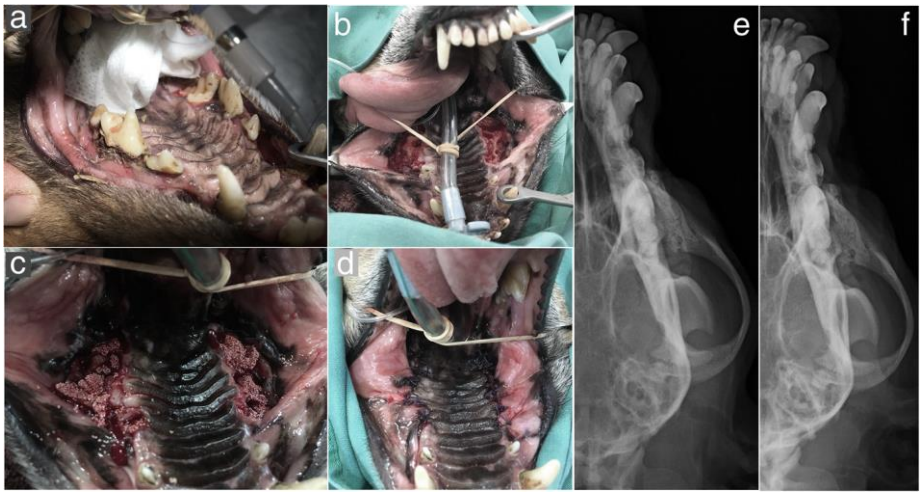


Fig. 8 Case #7 - Visual examination of the oral cavity affected by severe periodontal disease (**a**); Surgical extraction of all teeth caudal to the 1st maxillary pre-molars, resulting in exposed maxillary alveolar cavities (**b**), that were filled with Bonelike®(**c**) and covered by a mucosal flap (**d**); Postoperative control 12 weeks after exodontic procedure and filling of the defect using Bonelike® (**e**: right maxilla, **f**: left maxilla)

In clinical case #12, a 5-year-old female feline presented with periodontitis and several roots resorption. Clinically, the patient had developed progressive dysphagia. Conservative medical treatment with antibiotic and non-steroidal anti-inflammatory drugs was ineffective. Extensive maxillary tooth extraction was performed, and the alveolar cleft reconstructed with Bonelike®. An excellent functional recovery was observed and good behaviour recuperation was described (Fig. 13).

In clinical cases #13 and #14, feline patients presented with mandibular fractures. Treatment courses adopted were similar to those reported for the canine patients in Cases #3 and #4, through orthopaedic reduction and fixation of the fractures in combination to Bonelike®

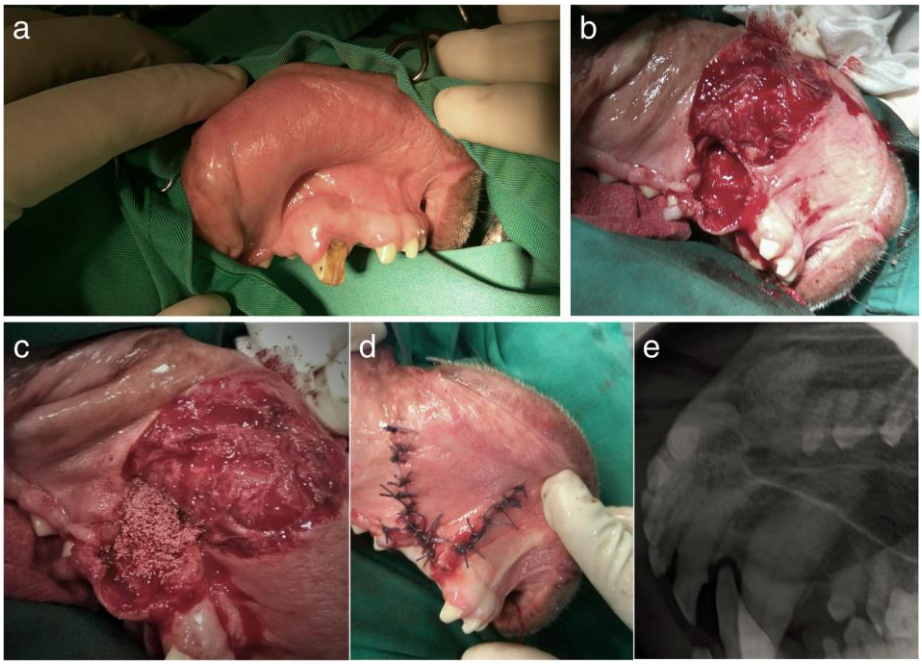


Fig. 9 Case #8 - Visual examination of the oral cavity and identification of the fractured canine tooth (**a**); Exposed maxillary alveolar cavity after tooth removal (**b**), that was filed with Bonelike®(**c**) and covered by a mucosal flap (**d**); Postoperative control 12 weeks after exodontic procedure and filling of the defect using Bonelike®(**e**)



Fig. 10 Case #9 - Visual examination of the oral cavity and identification of the fractured maxillary canine tooth (**a**); Exposed maxillary alveolar cavity after tooth removal that was covered by a mucosal flap (**b**); Preoperative control (**c**); Postoperative control after exodontic procedure, depicting the incomplete filling of the defect using Bonelike® (**d**)

grafting of the bone gaps. Both patients recovered well with satisfactory radiographic controls, indicating adequate bone healing. Fixation apparatus were removed 12 weeks after surgery.

Results

A series of 14 cases is herein reported, exploring the suitability of Bonelike® spherical granules of 250–500 μm diameter, in different types of bone defects in small animal clinical practice. In the detailed applications, Bonelike® was employed as bone substitute and to complement to traditional procedures, such as internal and external fixation of fractures, and iatrogenic bone voids.

Between the years of 2017 and 2018, the described veterinary clinical applications were performed in 10 canine and 4 feline patients, aged from 1 to 16 years (mean 7 years, median 8.5 years). These surgical procedures included Bonelike® grafting on defects after mandibular fractures with loss of bone and/or mandibular and maxillary tooth extractions (8 canine and 4 feline patients). Bonelike® applications described in the present manuscript, also included non-union fractures in long appendicular bones (2 canine patients).

Cases #1 and #2 presented with non-union defects of appendicular long bones, in canine patients, after previous surgical failures. Both resulted in good functional recovery after 12 weeks with the application of Bonelike® simultaneously to surgical reduction with osteosynthesis plates. Regarding maxillary and mandibular bone defects, 8 clinical cases were presented in canine and 4, in feline patients. Cases #3–4, and #13–14 were traumatic mandibular fractures in canine and feline patients, respectively. All were treated with external fixation except for case #3, in which an osteosynthesis plate was applied to the mandible. The remaining cases comprise Bonelike® application in alveolar defects, secondary to singular and multiple tooth extractions, to prevent oronasal fistula and imminent fractures. Through radiographic and physical examination, fracture consolidation was confirmed in all cases before 12 weeks.

Previous studies had proved Bonelike® biocompatibility in hard tissue applications [7], and no adverse systemic or local tissue reaction to Bonelike® were reported herein. Additionally, there was no record of post-operative infection, foreign body reaction or tear of the mucosa, regardless of the grafted defect size. Radiographic evaluation and clinical examination were performed in the follow-up periods in order to assess the efficacy of the surgical treatments associated to the bone substitute application. In all clinical cases, postoperative radiographic controls indicated high patterns of bone regeneration. All bone defects and extraction socket sites healed uneventfully for up to 12 weeks. At this point, the soft tissues

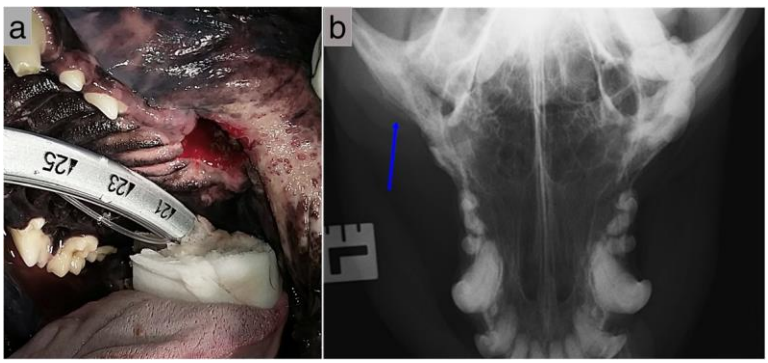


Fig. 11 Case #10 - Visual examination of the oral cavity and the molar tooth absence (**a**); Postoperative control 8 weeks after surgical filling of the defect using Bonelike® (**b**)

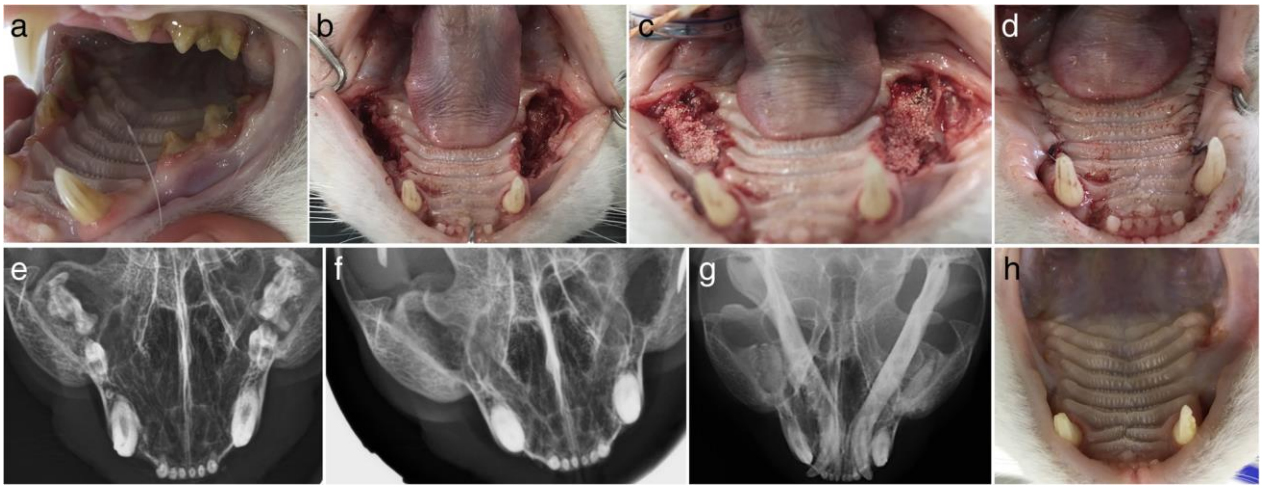


Fig. 12 Case #11 - Visual examination of the oral cavity evidencing periodontal disease and stomatitis-gingivitis complex (**a**); Exposed maxillary alveolar cavities after molar and premolar extraction (**b**), that were filed with Bonelike®(**c**) and covered by a mucosal flap (**d**); Preoperative radiographic control; **f**: Intraoperative radiographic control after tooth extraction (**e**); Postoperative control 6 weeks after surgical filling of the defect using Bonelike®(**g**); Visual examination of the oral cavity 6 weeks after surgery (**h**)

enclosing the surgical sites had a healthy appearance and physiological consistency. In all odontological cases, grafts were stable and surrounded by healthy mucosa, contributing to a total functional recovery.

The radio-opacity of the Bonelike® in the immediate postoperative radiographic controls enabled its differentiation from the native bone tissue, and allowed us to accompany its integration with the surrounding tissues (that resulted in attenuation and diffusion of the grafted cavities, and progressive integration on native bone radiodensity). Nevertheless, Bonelike® presence was still evidenced throughout the follow-up period, with increased opacity. This observation is particularly denoted in Case #6, where Bonelike® was applied

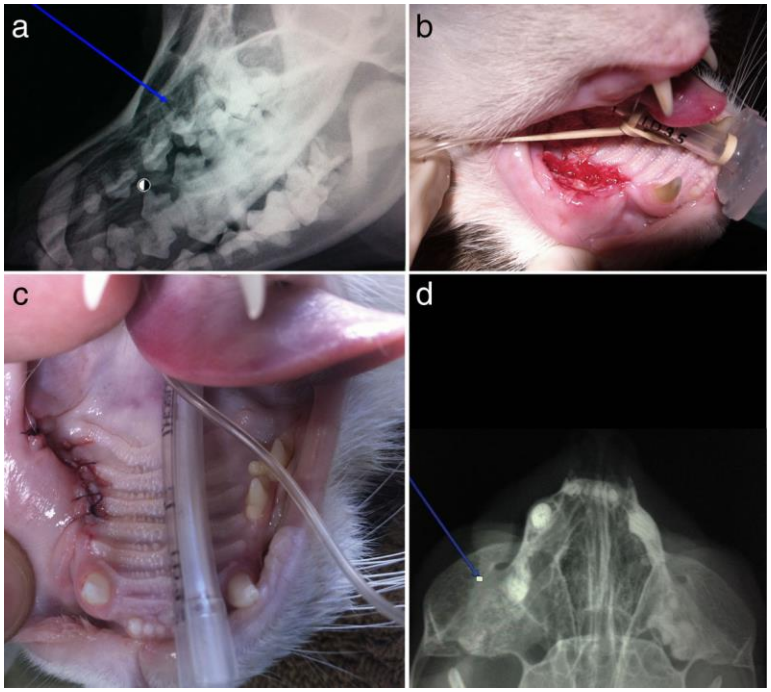


Fig. 13 Case #12 -Radiographic study evidencing dental root resorption (blue arrow) (**a**); Small alveolar defect after premolar unilateral exodontia (**b**) that was filed with Bonelike® and covered by a mucosal flap (**c**); Postoperative control 4 weeks after surgical filling of the defect using Bonelike® (blue arrow) (**d**)

unilaterally and a comparable contra-lateral defect was left unfilled.

Most importantly, legal tutors of the presented patients reported significant improvement on the quality of life of their pets, recovering previously impaired functions (weight-bearing in the recovering limb or gripping and/or mastication capacity). The most relevant point reported by the legal tutors was the absence of pain. Using the empiric adapted functional recovery scale, clinical recovery was classified as excellent (80%) in most cases, whereas 20% were classified as good.

Unfortunately, tutor compliance with follow-up scheduling was suboptimal, leading to the inability to further accompany bone-Bonelike® interactions and long-term outcomes.

Discussion

The physiologic reaction to a fracture is a spontaneous sequence of events briefly resumed as initial inflammation, followed by soft callus formation, hard callus formation, and ultimately bone remodeling [21]. When this natural process does not occur, as in the case of fracture non-union or large-scale traumatic bone injury, surgical intervention is warranted [22]. An insufficient blood supply and infection of the callus or the surrounding tissue or even systemic diseases can bare further negative effects on bone regeneration, resulting in a non-union [23]. The use of bone grafts is recommended in several surgical situations where the healing is difficult to achieve [24]. The consensual 'gold standard' graft remains the autograft, which does not induce immunological reactions and has the ability to provide osteoinductive growth factors, osteogenic cells, and acts as structural scaffold to new bone ingrowth [2, 22, 25]. However, this procedure is associated with prolonged anesthetic times, limited availability, donor site morbidity (pain, intra-operative blood loss and risk of stress fracture), risk of local infection and predisposition to failure [17]. Other issue is the limited amount of bone graft that must be collected from the animal and the viability of the cells after harvesting that limits the application to critical defects [25]. To address these problems, synthetic bone substitutes overcome some of the disadvantages listed for the autografts and can be used to fill critical voided spaces. These osteoinductive materials can be stored easily [22, 26, 27].The present case series report demonstrates Bonelike® spherical granules as an alternative to bone grafting techniques in the recovery of those patients, restoring the bio functionality of the affected area in small animals, in a similar manner to previously reported in maxillofacial surgery in human patients [28]. Bonelike® is an excellent ceramic scaffold to promote bone regeneration processes. It is made from an inorganic, nonmetallic material that can possess a crystalline structure [22]. Bonelike® contains a great proportion of HA, which is the main mineral component of the naturally occurring bone [29]. The complex composition of Bonelike® that combines HA, TCP and bioglass results in a tailored degradation rate, that accommodates new bone ingrowth while maintaining the grafted area integrity (the TCP phase has as faster degradation rate than the HA and bioglass phases) [7, 14].

In Case#1 a complimentary approach was adopted, due to the extension of the bone defect (and its critical nature), and an autologous cancellous bone graft was combined with Bonelike® spherical granules, demonstrating that compromise between bone and biomaterial grafting techniques may boost the clinical outcomes of complex cases, probably due to the addition of important growth factors and biomolecules present in the bone that promote the bone regeneration of the defects.

The remaining reported defects were of sub-critical size, and adequate bone healing was expected in defined periods of time (mandibular and maxillary bones 3-4 weeks; appendicular bones 8-12 weeks). Nevertheless, the application of the biomaterial filler aimed at the acceleration of the mineralization of the bone voids, thus decreased the risk for postoperative fractures associated with resulting bone fragility.

The maintenance of small granular biomaterial within a cavitary defect is often a challenge and mixing with an adjuvant matrix is often required. Mixing of Bonelike® with autologous peripheral blood and or platelet derived plasma achieved the desired effect in the detailed cases, avoiding biomaterial leakage. Additionally, the cellular populations enclosed within the formed clots are source for bioactive factors that activate intrinsic healing mechanisms at the defect site, and are proposed to further contribute to improved regeneration [30]. Other matrices would be interesting for the combined application, such as surgical fibrin glue, but associated costs may prevent widespread utilization, especially in the Veterinary Medicine field [31]. In the specific application of Bonelike® in oral surgery, mixture with the haemoderived matrix was reinforced by a mucosal flap, to further stabilize the Bonelike® spherical granules in the cavity and to protect the site from foreign bodies' penetration (food, as an example). Currently, the goal was to fill the alveolar defect left by exodontic procedures. In similar applications in human patients, the target is the preparation of a congruent cavity in the alveolar process, thus promoting the integration between bone and future placed dental implant [32].

This surgical approach using a synthetic bone substitute, may be additionally combined with cell-based therapies, to potentiate the benefits of osteoregenerative biomaterials in orthopedic and oral surgery [33]. The combination of such cell-based therapies (dental pulp

stem cells, for example) results in enhanced and accelerated regeneration processes [31].

Nevertheless, further prospective randomized clinical trials are essential to substantiate preliminary clinical case reports and provide quantitative and comparative data between the available and recently developed therapeutic strategies [9, 23].

Conclusions

Autologous bone remains the gold standard grafting substrate available for bone fusions for small gaps and critical defects, but ceramic-based biomaterials, such as Bonelike® arise as a very adequate candidate biomaterial for application in orthopedic and oral surgery, in both humans and veterinary clinical scenarios. The present report details a series of 14 small animal clinical cases, including appendicular bone defects and maxillary / mandibular bone defects. In all clinical cases, Bonelike® application complement the recommended surgical techniques, and resulted in enhanced bone healing and total functional recovery. The positive defect healing and lack of tissue and systemic adverse effects observed allows us to also hypothesise Bonelike® as a structural scaffold for new-bone ingrowth. This strategy is available to be used in small animals as a space filler, and in association with standard orthopaedic and odontological procedures, thus promoting accelerated recoveries. This procedure improves the animals' quality of life, decreasing pain and post-operative recovery period, as well as increasing bone stability improving positive clinical outcomes.

Nevertheless, clinical trials are required in Veterinary Medicine to adequately assess the outcome of the novel treatment options, establishing the most appropriate treatment protocols for each clinical presentation.

Abbreviations

HA: Hydroxyapatite; TCP: Tricalcium phosphate

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JMC performed almost all the experimental surgeries, researched and wrote the manuscript. JDS, ACS and AB carried out preparation and synthesis the Bonelike®. POP, JR, MLF performed some of the experimental surgeries. ARC, ALL, MVB, LMA, CM, AA and SSP organized this research and helped to write the manuscript. ACM conceived the study, participated in its design and coordination, and helped to draft the manuscript and revised the data and the manuscript. ACM was also responsible for the funding support. All author read and approved the final manuscript.

Ethics approval and consent to participate

All the animal testing procedures were in conformity with the Directive 2010/63/EU of the European Parliament and the Portuguese DL 113/2013. All the procedures were approved by the ICBAS-UP Animal Welfare Organism of the Ethics Committee (ORBEA) and by the Veterinary Authorities of Portugal (DGAV). Humane end points were followed in accordance to the OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000), and adequate measures were taken to minimize pain and discomfort considering human endpoints for animal suffering and distress. The interaction of Bonelike® in bone tissue was evaluated in 10 dogs and 4 cats.

Consent for publication

Not applicable.

Competing interests

The authors report no financial or other conflicts relates to this report. The authors do not have conflict of interest with any of the materials or companies described in the manuscript. Although A Brandão is an employee of Biosckin, Molecular and Cell Therapies, S.A. this does not alter the adherence to policies on sharing data and materials.

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References

1. Kamitakahara M, Ohtsuki C, Miyazaki T. Behavior of ceramic biomaterials derived from tricalcium phosphate in physiological condition. J Biomater Appl. 2008;23:197–212.
2. Bohner M. Resorbable biomaterials as bone graft substitutes. Mater Today. 2010;13:24–30.
3. Arvidson K, Abdallah B, Applegate L, Baldini N, Cenni E, Gomez-Barrena E, Granchi D, Kassem M, Konttinen Y, Mustafa K. Bone regeneration and stem cells. J Cell Mol Med. 2011;15:718–46.

4. Bai X, Gao M, Syed S, Zhuang J, Xu X, Zhang XQ. Bioactive hydrogels for bone regeneration. Bioact Mater. 2018;3:401–17.
5. Ferracini R, Martinez Herreros I, Russo A, Casalini T, Rossi F, Perale G. Scaffolds as structural tools for bone-targeted drug delivery. Pharmaceutics. 2018;10.
6. He W, Fan Y, Li X. Recent research progress of bioactivity mechanism and application of bone repair materials. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi. 2018;32:1.
7. Atayde LM, Cortez PP, Afonso A, Santos M, Maurício AC, Santos JD. Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike®) on osteoregeneration. J Biomed Mater Res B Appl Biomater. 2015;103:292–304.
8. Santos J, Hastings G, Knowles J. Sintered Hydroxyapatite compositions and methods for the preparation thereof. European patent WO 0068164. 1999.
9. Pinto PO, Atayde LM, Campos JM, Caseiro AR. Therapeutic strategies for bone regeneration : the importance of biomaterials testing in adequate animal models. In: Tiwari A, Alenezi M, Jun S, editors. Scrivener publishing; 2016. p. 275–319.
10. Gutierres M, Dias A, Lopes M, Hussain NS, Cabral A, Almeida L, Santos J. Opening wedge high tibial osteotomy using 3D biomodelling Bonelike® macroporous structures: case report. J Mater Sci Mater Med. 2007;18:2377–82.
11. Pavan Kumar G, Jaya Kumar A, Krishnanjaneya Reddy P, Nandyala SH, Lopes MA, Santos JD. Application of glass reinforced hydroxyapatite composite in the treatment of human intrabony periodontal angular defects–two case reports. Trans Tech Publ. 2010:93–101.
12. Hussain NS, Lopes MA, Maurício AC, Ali N, Fernandes MH, Santos JD. 709 Bonelike® Graft for Regenerative Bone Applications. In Surface Engineered Surgical Tools and Medical Devices. Edited by Jackson MJ, Ahmed W: Springer US; 2007;477–512.
13. Oliveira JM, Miyazaki T, Lopes MA, Ohtsuki C, Santos JD. Bonelike®/PLGA hybrid materials for bone regeneration: preparation route and physicochemical characterisation. J Mater Sci Mater Med. 2005;16:253–9.
14. Cortez PP, Atayde LM, Silva MA, Fernandes MH, Afonso A, Lopes MA, Mauri AC. Characterization and preliminary in vivo evaluation of a novel modified hydroxyapatite produced by extrusion and spheronization techniques. J Biomed Mater Res B Appl Biomater. 2011;99:170–9.
15. Lopes M, Santos JD, Monteiro F, Knowles J. Glass-reinforced hydroxyapatite: A comprehensive study of the effect of glass composition on the crystallography of the composite; 1998.
16. Shirosaki Y, Botelho CM, Lopes MA, Santos JD. Synthesis and characterization of chitosan-silicate hydrogel as resorbable vehicle for bonelike® bone graft. J Nanosci Nanotechnol. 2009;9:3714–9.
17. Pinto P, Atayde L, Campos J, Caseiro A, Pereira T, Mendonça C, Santos J, Maurício A. Therapeutic strategies for bone regeneration: the importance of biomaterials testing in adequate animal models. Adv Compos Mater. 2016:275–319.
18. Izumisawa Y, Seno T, Abe R, Miyoshi K, Maehara S, Wakaiki S, Kushiro T, Umar MA, Tsuzuki K, Yamashita K, Hayashi S. Axial correction of pes varus by transverse-opening wedge osteotomy and T-plate fixation with beta-tricalcium phosphate (beta-TCP) transplantation in dachshunds. J Vet Med Sci. 2005;67:437–40.
19. Ulery BD, Nair LS, Laurencin CT. Biomedical applications of biodegradable polymers. J Polym Sci B Polym Phys. 2011;49:832–64.
20. Franch J, Diaz-Bertrana C, Lafuente P, Fontecha P, Durall I. Beta-tricalcium phosphate as a synthetic cancellous bone graft in veterinary orthopaedics: a retrospective study of 13 clinical cases. Vet Comp Orthop Traumatol. 2006; 19:196–204.
21. Lopes D, Martins-Cruz C, Oliveira MB, Mano JF. Bone physiology as inspiration for tissue regenerative therapies. Biomaterials. 2018;185:240–75.
22. Khan Y, Yaszemski MJ, Mikos AG, Laurencin CT. Tissue engineering of bone: material and matrix considerations. JBJS. 2008;90:36–42.
23. Janicki P, Schmidmaier G. What should be the characteristics of the ideal bone graft substitute? Combining scaffolds with growth factors and/or stem cells. Injury. 2011;42:S77–81.
24. Şen C, Balci HI, Celiktaş M, Ozkan C, Gulsen M. Definitive surgery for open fractures of the long bones with external Fixatıon. In: Basic techniques for extremity reconstruction: Springer; 2018. p. 107–28.
25. Rogers GF, Greene AK. Autogenous bone graft: basic science and clinical implications. J Craniofac Surg. 2012;23:323–7.
26. Finkemeier CG. Bone-grafting and bone-graft substitutes. JBJS. 2002;84:454–64.
27. Lobato J, Hussain NS, Botelho C, Maurício A, Afonso A, Ali N, Santos J. Assessment of bonelike® graft with a resorbable matrix using an animal model. Thin Solid Films. 2006;515:362–7.
28. Sousa R, Lobato J, Maurício A, Hussain N, Botelho C, Lopes M, Santos J. A clinical report of bone regeneration in maxillofacial surgery using bonelike® synthetic bone graft. J Biomater Appl. 2008;22:373–85.
29. Anandan D, Jaiswal AK. Synthesis and characterization of human bone-like hydroxyapatite using Schiff's base. Ceram Int. 2018;44:9401–7.
30. Schuckert KH, Jopp S, Osadnik M. The use of platelet rich plasma, bone morphogenetic protein-2 and different scaffolds in oral and maxillofacial surgery - literature review in comparison with own clinical experience. J Oral Maxillofac Res. 2011;2:e2.
31. Campos J, Sousa A, Caseiro A, Pedrosa S, PO P, MV B, Amorim I, Santos J, Pereira T, Mendonça C, et al. Dental Pulp Stem Cells and Bonelike® for Bone Regeneration in Ovine Model. Regenerative Biomaterial (IN PRESS) 2018.
32. Berglundh T, Lindhe J. Healing around implants placed in bone defects treated with Bio-Oss®. An experimental study in the dog. Clin Oral Implants Res. 1997;8:117–24.
33. Caseiro AR, Alvites R, Pedrosa SS, Miguel J, Reis IL, Santos JD, Mendonça C, Atayde M, Maurício AC. The potential clinical application of Mesenchymal stem cells from the dental pulp (DPSCs) for bone regeneration. In Frontiers in Stem Cell and Regenerative Medicine Research (Atta-ur-Rahman, FRS, Shazia Anjum). India: Bentham Science; 2017. p. 21–70.

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Chapter 10 – General Final Considerations

In order to systematically complete all the experimental work developed during my PhD thesis, I will present general considerations about:

About bone grafts considerations

The use of bone grafts is still recommended in several surgeries. Autografts continue to be an excellent tool in the management of certain bone defects because the results of cost-effective combination of biologic and mechanical properties are excellent. Autografts have the ability to provide osteoinductive growth factors and do not induce local nor systemic immunological reactions. An autografts also provides a scaffold for osteoprogenitor cells and other important viable cells for the regenerative process while avoiding disease transmission. It is still considered to be the technique of choice in nonunion or bone fusions for small gaps and in critical defects. Regardless of its unique characteristics, autografts are associated with several disadvantages such as prolonged anesthetic times, limited availability of bone graft that can be collected from the patient, the viability of the cells after harvesting, pain, intra-operative blood loss, stress fracture, risk of local infection and predisposition to failure. The referred disadvantages of autografts have driven to the development of synthetic bone grafts for bone regeneration either in human or in veterinary medicine, leading to an enlargement of the need to use biomaterials.

Some synthetic bone substitutes have only osteoconductive properties if not combined with recombinant human growth factors or autografts. When these bone substitutes are combined with growth factors, may have equal biologic properties to autologous bone grafts but further research is required to determine their efficacy and cost-benefit profile.

About Bonelike® considerations

Bonelike® is a synthetic bone graft substitute consisting of the incorporation of P_2O_5-CaO glass-based system within the HA matrix of spherical granules with 250-500μm of diameter. This biomaterial overcomes some of the main disadvantages of traditional HA and the disadvantages listed for the autografts [91].

Bonelike® is composed of a modified HA matrix with α- and β-TCP secondary phases, resulting in higher solubility than single HA type of materials. The combination of the HA and the TCP allows to create ideal conditions because the bone graft is reabsorbed at a rate close to the formation of new bone. This fact creates better osteoinductive and osteoconductive proprieties that have been confirmed in experimental models of bone regeneration in sheep and recently its application in orthopedic and stomatology pathologies is being tested in small animal clinics. From the clinical point of view, the Bonelike® Spherical shape allows the surgeon to separately handle each bead with tweezers or even with a curette. It's a regular shape with some difficult of fixation inside the bone lose. Reducing the movement of the biomaterial in the damaged area is an important aspect that should be considered otherwise all the biomaterial can leave the defect [91] [335].

Spherical granules with 250-500μm allow the bone graft material to be delivered through small orifices, yet maintaining enough porosity to allow capillary action, cellular infiltration and vascular incorporation. It presents sites for cellular adhesion as well as the interconnectivity between pores necessary for the transport of nutrients, favoring cell adhesion and proliferation and consequently the growth of bone tissue, however it is possible to create a better Bonelike® if the shape of this biomaterial had a higher porosity.

Our research group is still studying a model of Bonelike® with macroporosity (granules with 1000μm to 2000μm). Larger granules will allow the migration of the cells through the interior of the biomaterial with the possibility of colonization in a much larger area, being therefore advantageous in comparison to Bonelike® Spherical [132]. Higher granulometry of Bonelike® should be easily shaped after contact with a biological fluid or autologous blood with minimal mixing and can be delivered via injection through delivery tubes of varying diameters while maintaining optimal porosity and pore size distribution for resorption, cellular infiltration and

impregnation. These implant formulations will offer easy-to-use doses of composite material and are a significant advancement over current bone graft systems for clinical applications [132].

About Bonelike® with DPSCs

DPSCs can be found in individuals of any age, and can be easily isolated from deciduous and definitive teeth, expanded and cryopreserved for further clinical application. These cells are capable of differentiating towards multiple lineages, present great potential for osteo-differentiation and for promoting the regeneration of bone tissue through the secretion of important bioactive molecules (so called secretome). DPSCs have been demonstrated to incorporate diverse biomaterial systems and promote mineral deposition both in vitro and in vivo, aiming at the reconstruction of osseous defects, in either experimental or clinical situations.

DPSCs can also be found and isolated from the dental pulp of many species other than humans, granting them potential to be implemented not only in human medicine but also in veterinary care practices, and in regenerative strategies for other organs and tissues, such as dental reconstruction and nervous system regeneration.

DPSCs can be isolated from the dental pulp of of healthy teeth, from young and adult individuals (from deciduous and definitive dentition) and can be considered an interesting alternative to bone marrow, adipose tissue and umbilical cord matrix MSCs.

The general screening assays for testing a scaffold are divided into different phases:

1) General toxicity (in vitro cytotoxicity tests);
2) Local tissue irritation (animal implantation);
3) Pre-clinical (proof of concept in an animal model);
4) Clinical evaluation (animals and human trials) [344].

The first phase was already described in other previous studies of our experimental group and Bonelike® showed to have an excellent behavior in terms of

toxicity over the tissues [335]. The in vitro studies with the association of Bonelike® with hDPCS have been performed and the manuscript is under preparation.

The *in vivo* evaluation was performed in the ovine model in order to analyze the filling of non-critical defects with Bonelike® (250 μm <Φ <500 μm) and hDPSCs, at different times of implantation of 30, 60 and 120 days. These studies allowed us to evaluate the evolution of bone healing, the quality of the new formed bone and the interaction of the biomaterial with the bone formed.

In the specific conditions used during these experimentation, regeneration using the biomaterial associated to the cellular system was better than the bone regeneration in defects that did not receive any biomaterial or even were just filled with Bonelike®. The analysis of the results of the post-mortem radiographs, histological and histomorphometric analysis demonstrated that bone defects filled with Bonelike® plus hDPSCs tend to have a better bone formation compared to the other experimental defects. Accelerated defect bridging in the biomaterial filled defects was observed. The biomaterial posed as both a stimuli and scaffold of newly-formed tissue penetration and is progressively embedded within the newly deposited matrix. The observed features confirms other authors' previous findings [73].

Biomaterial-treated defects showed faster bone maturation than controls, with lamellar bone formation and arise of Haversian systems. This phenomenon reflects the osteopromotion associated to the porous nature of the biomaterial, which facilitates cell anchoring and protein absorption and consequently better osteoconduction. Indeed, the microtomography (roughness) of the biomaterial has been demonstrated to be of the utmost importance to hDPSCs adhesion and osteopromotion [261], and that microconcave surfaces favour osteodifferentiation and formation of thick mineralized tissue by seeded cells, due to the resemblance to native bone trabecular spaces [345].

The spherical shape and the granulometry of Bonelike® has been previously identified as to naturally adapt circular pattern of native bone growth [73]. These characteristics also enhance cell migration, proliferation of native and delivered cells, as well as improving vascular invasion, resulting in optimized osteointegration and osteo-reabsorption.

These results are due to the fact that hDPSCs have a great potential for osteo-differentiation. These cells can differentiate into osteoblasts / osteocytes, thus producing a mineralized matrix and can also produce locally, growth factors, cytokines and other molecules that promote the regeneration process and the inflammation, even by recruitment of different cells to the injury site. Furthermore, hDPSCs have been demonstrated to secrete an extensive array of bioactive factors (growth factors and cytokines) [346] that modulate the activity of native cells and inflammatory populations at lesion sites and therefore unleash the regenerative response. The addition of hDPSCs to the therapeutic system resulted in higher bone growth and accelerated defect bridging, ensuring contact of bone edges from the centre to the endosteal surface of the bone. Since bone regeneration in these cases is more rapid and effective, at a clinical level these may reduce the risk of the appearance of post-operative premature spontaneous fractures and improve the quality of the bone *callus* and the neo-formed bone [224].

Through the histological analysis of the samples with Solochrome Cyanine R it was possible to observe an initial absorption with low degradation of the biomaterial after 30 days of implantation, while after 120 days of implantation it was possible to observe absorption and high degradation. Thus, Bonelike® 250-500 μm presented a pattern of resorption with the invasion of neo-formed bone into the particle. Initially, there was an increase in the intra-porosity of the biomaterial, thus forming spaces within the particles. With increasing implantation time, these spaces were replaced by bone. Through the EDS analysis it was possible to confirm that the material formed within the Bonelike® was effectively neo-formed bone [193].

In the histomorphometric analysis the results evidenced the decrease of the percentage of Bonelike® throughout the times of implantation. Once again it was confirmed that Bonelike® is considered bioreabsorbable since the material dissolves / reabsorbs and is gradually replaced by neo-formed bone [193].

One of the difficulties found was the maintenance of the biomaterial granules and hDPSCs at the site of the defect without falling into the bone marrow. To overcome this problem, Tisseel® was used as a sealant and cell-based therapy vehicle, however, in the histological analyses, even using this mixture, there was still some material in the bone marrow. It was observed that Bonelike® had a poor capacity to

remain stable in larger defects and the use of shaped blocks to retain the bone substitute in place may be a future advantage when dealing with critical defects.

Another concern in the *in vivo* test was the difficulty in using the same amount of biomaterial in each bone defect, which could influence the results. Also, it is also necessary to take into account that when we tested in different sheep, we were confronted with a great individual variability.

About Bonelike® with DPSCs in ovine model

The ovine model presented in bone repair include a representation in which regular healing processes are compromised without presence of a critical-sized defect site. In a critical-sized segmental defect models bridging of the respective defect does not occur despite a sufficient biological microenvironment due to the removal of critical amounts of bone substance. In contrast, in a true non-union deficient signalling mechanisms, biomechanical stimuli or cellular responses may prevent defect healing rather than the defect size.

The model proposed use a cortical access that allows to create 10 to 12 defects per sheep, in the same bone with equal conditions of loading, bone structure and amounts of overlying soft tissue. Therefore, it is possible to perform serial sampling in the same animal with the same environmental conditions. The surgical technique is relatively simple: to access the femur, only the skin and *fascia lata* are incised, followed by a blunt dissection without risk of damaging important structures. After that 5 mm size defects are able to create in both femurs [82, 193].

Despite being a non-critical size, the healing process is studied. This model, allowing the performance of multiple serial analyses of a biomaterial within the same in vivo conditions; this feature is very important on the initial screening of a biomaterial *in vivo* behaviour, enabling to choose the most suitable chemical composition and the best format of the biomaterial.

The Bonelike® may also have a unit dose, in terms of quantity, for each hole. It means the quantity of each dose of Bonelike® used should be the same in all small defects created in the femur during the experience in sheep. For repairing bone voids, we wet the bone graft material with a predetermined amount of autologous blood. In

a preferred embodiment, the ratio for wetting the bone graft to obtain the desired mass with an appropriate viscosity for injecting and repairing bone voids is supposedly 2.0 cc- 2.5cc of blood per one sample of bone graft material.

In the *in vivo* evaluation, the addition of hDPSCs to the therapeutic system resulted in higher bone growth and accelerated defect bridging, ensuring contact of bone edges. Immunohistochemical staining also denoted increased mature lamellar bone content in Bonelike® treated defects, with improved histological features recovery. The improvement and acceleration of bone regeneration events is particularly evident at the earliest times of regeneration.

A multidisciplinary approach will be required to improve implanted cell survival and to ensure prompt vessel ingrowth into the biomaterial via careful selection of structure and shape, together with addition of cytokines and growth factors.

In future works, it will be important to carry out further biomaterial characterization tests such as, for example, mercury porosimetry to obtain data like pore size distribution, total surface area, total pore volume, mean pore diameter and apparent and actual mass of the sample. It will also be important to discover a fixation medium that does not exhibit the inhibitory effect on cell viability and proliferation, in order to test in Bonelike® direct assays.

About Bonelike® in clinical cases

In the clinical trial, the Bonelike® bone graft substitute has shown to be effective for treatment of bone lesion defects but its mechanical, histological and radiographic characteristics have not been studied in direct comparison with a conventional treatment such as cancellous allograft bone.

This study revealed an optimized therapeutic modality for regeneration of non-critical-sized bone defects in large bones and it has two major advantages including acceptable therapeutic efficacy and reliability. If these encouraging results observed in the animal ovine model affirm to be consistent in human and other mammals, this could open new horizons to the development of clinical applications. The present study suggests that the osteoinductive scaffolds, especially Bonelike® with the incorporation of hDPSCs, enhance angiogenesis and bone repair in comparison to conventional scaffolds, which were proved by in vivo and in vitro analyses. Therefore,

due to perfect new bone formation along with high in vivo biodegradability of this scaffold, it can be introduced as a promising alternative to autografts [193, 225].

The combining of Bonelike® with hDPSc finds particular suitability in applications that require regularly shaped and no-critical sized bone void defects to be filled. Bonelike® has a highly bioactive behavior, demonstrated in several *in vitro* and *in vivo* studies together with some clinical trials. Granular forms of this biomaterial were applied in the presented cases applications demonstrating the benefits of its properties for bone regeneration. However, these studies also highlighted the need to develop methods towards a fast consolidation of Bonelike® when applied in unstable sites, along with an improvement of its handling properties and injectability if used in minimally invasive surgical techniques.

Moreover, Bonelike® is useful as an injectable for filling bone defects of various shapes and sizes in orthopedic, dentistry or maxillo-facial surgery applications of small animals as void space filler and in association with standard orthopedic and dental procedures of stabilization, promoting a faster bone fusion without any local or systemic adverse reaction. This procedure improves the animal's quality of life, decreasing the pain and increasing the bone stability with positive clinical outcome and decreasing the post-operative period recovery [197].

Finally, it is possible to admit that with the panoply of studies, possibilities and future developments, it will be easier to adapt the treatments using biomaterials and cells-therapies to animals and human's patients in the context of an advanced regenerative medicine.

In order to improve the quality of bone regeneration, several clinical cases were reported. In various small animal cases such as dentistry, orthopaedic and maxillo-facial surgical cases, exists the need to use bone graft substitutes instead of autografts. For instance, following tooth extraction, the alveolar bone supporting the tooth undergoes constant atrophy. It is showed that a marked reduction of the height of the alveolar ridge consistently occurred following tooth extraction. For an improved functional result, it is necessary to preserve the alveolar bone volume after tooth extraction to facilitate the act of chewing in pets and the subsequent placement of dental implants in humans. It can be supposed that bone augmentation procedures are effective in promoting bone fill and defect resolution in post-extraction sites,

avoiding spontaneous fractures in small animals and perhaps will be interesting to study the application of dental implants in animals.

The osteoinductive and osteoconductive proprieties have been confirmed before in experimental animal models of bone regeneration in sheep and recently its application was performed in orthopaedic, maxillofacial and dentistry cases in small animal clinic at HVUC and UP-VET.

Conclusion

In conclusion of this experimental research and based on the results obtained, using a mono-cortical non-critical defect of the femur of an ovine model, the addition of osteogenic cells (hDPSCs) to biomaterial scaffolds (Bonelike® 250-500μm) can enhance the results of histomorphometric bone regeneration and osteointegration in bone defects.

This study demonstrated that hDPSCs are capable to be isolated and cryopreserved previously to experimental and clinical application, and therefore the scaffold consisting of Bonelike® and hDPSCs would be promising in the regeneration of bone. Although hDPSCs seem to be a favorable cellular therapy for bone regeneration in the referred model, additional research is needed in the area of orthopedic, dentistry and maxillo-facial surgery.

In the clinical cases presented that included companion animals, Bonelike® demonstrated to be an adequate bone graft substitute and has showed to be effective in the treatment of bone defects with acceptable recovery and satisfactory mechanical and radiographic results.

Regarding the conclusions of my PhD work, my future perspective is that hDPSCs along with larger Bonelike® spherical granules would be an excellent cell-based therapy, preferably as an autologous or allogeneic source in the context of tissue engineering for bone regeneration. Cell-based strategies may be used for critical, large and compromised bone defects, where surgical treatments are limited and often are not successful.

Bonelike® is a commercial synthetic bone graft with numerous applications in tissue engineering and has been demonstrated has a potential scaffold for DPSCs.

In our research group, more studies are currently being performed to better understand how these cell therapies can be effectively used *in vivo*.

Compliance with ethical standards

The author declare that he has no conflict of interest.

Bibliography

[1] R. J. Estes and M. J. Sirgy, "Global Advances in Quality of Life and Well-Being: Past, Present, and Future," *Social Indicators Research,* pp. 1-28, 2018.

[2] C. European, "Demography Report - Short Analytical Web Note," Luxembourg9789279466113, 2015, Available: http://ec.europa.eu/eurostat/documents/3217494/6917833/KE-BM-15-003-EN-N.pdf/76dac490-9176-47bc-80d9-029e1d967af6.

[3] M. Bohner, "Resorbable biomaterials as bone graft substitutes," *Materials Today,* vol. 13, no. 1-2, pp. 24-30, 2010.

[4] Y. B. Yingzhi Gu, Dongliang Zhang, "Osteogenic stimulation of human dental pulp stem cells with a novel gelatin-hydroxyapatite-tricalcium phosphate scaffold," *JOURNAL OF BIOMEDICAL MATERIALS RESEARCH* vol. 00A, no. 00, 2018.

[5] A. S. Neto and J. M. Ferreira, "Synthetic and Marine-Derived Porous Bone Graft Substitutes," *Bone,* vol. 21, p. 22, 2018.

[6] L. M. Redondo *et al.*, "Repair of maxillary cystic bone defects with mesenchymal stem cells seeded on a cross-linked serum scaffold," *Journal of Cranio-Maxillofacial Surgery,* vol. 46, no. 2, pp. 222-229, 2018/02/01/ 2018.

[7] W. Wang and K. W. K. Yeung, "Bone grafts and biomaterials substitutes for bone defect repair: A review," *Bioactive Materials,* pp. 1-24, 2017.

[8] A. Decambron *et al.*, "Low-dose BMP-2 and MSC dual delivery onto coral scaffold for critical-size bone defect regeneration in sheep," (in eng), *J Orthop Res,* vol. 35, no. 12, pp. 2637-2645, Dec 2017.

[9] J. P. Bilezikian, L. G. Raisz, and G. A. Rodan, "Structure and Development of the Skeleton," in *Principles of Bone Biology*, vol. 1, A. Press, Ed. United States Of America: Academic Press, 2002, pp. 3-15.

[10] J. A. McGovern, M. Griffin, and D. W. Hutmacher, "Animal models for bone tissue engineering and modelling disease," (in eng), *Dis Model Mech,* vol. 11, no. 4, Apr 23 2018.

[11] A. M. Weatherholt, R. K. Fuchs, and S. J. Warden, "Specialized connective tissue: bone, the structural framework of the upper extremity," *Journal of hand therapy : official journal of the American Society of Hand Therapists,* vol. 25, no. 2, pp. 123-132, 2012.

[12] A. A. El-Rashidy, J. A. Roether, L. Harhaus, U. Kneser, and A. R. Boccaccini, "Regenerating bone with bioactive glass scaffolds: A review of in vivo studies in bone defect models," (in eng), *Acta Biomater,* vol. 62, pp. 1-28, Oct 15 2017.

[13] C. J. Kowalczewski and J. M. Saul, "Biomaterials for the Delivery of Growth Factors and Other Therapeutic Agents in Tissue Engineering Approaches to Bone Regeneration," (in eng), *Front Pharmacol,* vol. 9, p. 513, 2018.

[14] A. M. Weatherholt, R. K. Fuchs, and S. J. Warden, "Specialized Connective Tissue: Bone, the Structural Framework of the Upper Extremity," *Journal of Hand Therapy,* vol. 25, no. 2, pp. 123-132, 2012/04/01/ 2012.

[15] A. Oryan, S. Alidadi, A. Moshiri, and N. Maffulli, "Bone regenerative medicine: classic options, novel strategies, and future directions," *Journal of Orthopaedic Surgery and Research,* vol. 9, pp. 18-18, 03/17

09/10/received

02/20/accepted 2014.

[16] R. Miron *et al.*, *Osteoinductive potential of 4 commonly employed bone grafts*. 2016.

[17] D. G. Evans, S. M. Goldman, and R. T. Kronengold, "Devices and methods for treating defects in the tissue of a living being," ed: Google Patents, 2018.

[18] B. Akar *et al.*, "Large Animal Models of an In Vivo Bioreactor for Engineering Vascularized Bone," (in eng), *Tissue Eng Part B Rev,* vol. 24, no. 4, pp. 317-325, Aug 2018.

[19] C. Schopper *et al.*, "HA/TCP compounding of a porous CaP biomaterial improves bone formation and scaffold degradation--a long-term histological study," (in eng), *J Biomed Mater Res B Appl Biomater,* vol. 74, no. 1, pp. 458-67, Jul 2005.
[20] E. H. Schemitsch, "Size Matters: Defining Critical in Bone Defect Size!," (in eng), *J Orthop Trauma,* vol. 31 Suppl 5, pp. S20-s22, Oct 2017.
[21] P. O. Pinto, L. M. Atayde, J. M. Campos, and A. R. Caseiro, "Therapeutic Strategies for Bone Regeneration : The Importance of Biomaterials Testing in Adequate Animal Models," A. Tiwari, M. Alenezi, and S. Jun, Eds.: Scrivener Publishing, 2016, pp. 275-319.
[22] J. C. Reichert *et al.*, "A tissue engineering solution for segmental defect regeneration in load-bearing long bones," (in eng), *Sci Transl Med,* vol. 4, no. 141, p. 141ra93, Jul 4 2012.
[23] M. Jager, M. Sager, S. Lensing-Hohn, and R. Krauspe, "The critical size bony defect in a small animal for bone healing studies (II): implant evolution and surgical technique on a rat's femur," (in eng), *Biomed Tech (Berl),* vol. 50, no. 5, pp. 137-42, May 2005.
[24] A. Houssaye, K. Waskow, S. Hayashi, R. Cornette, A. Lee, and J. Hutchinson, "Biomechanical evolution of solid bones in large animals: a microanatomical investigation," *Biological Journal of the Linnean Society,* no. 117, pp. 350–371, 2016.
[25] C. Clajus, F. Blasi, T. Welte, M. Greer, T. Fuehner, and M. Mantero, "Therapeutic approach to respiratory infections in lung transplantation," *Pulm Pharmacol Ther,* Jul 17 2014.
[26] K. M. Tobias and S. A. Johnston, "Veterinary Surgery Small Animal," vol. 1, E. Saunders, Ed., ed, 2012, p. 557.
[27] A. J. Salgado, O. P. Coutinho, and R. L. Reis, "Bone Tissue Engineering: State of the Art and Future Trends," *Macromolecular Bioscience,* vol. 4, no. 8, pp. 743-765, 2004.
[28] J. Gasser and M. Kneissel, "Bone Physiology and Biology," 2017, pp. 27-94.
[29] A. K. Nair, A. Gautieri, S. W. Chang, and M. J. Buehler, "Molecular mechanics of mineralized collagen fibrils in bone," (in eng), *Nat Commun,* vol. 4, p. 1724, 2013.
[30] J. Y. Rho, L. Kuhn-Spearing, and P. Zioupos, "Mechanical properties and the hierarchical structure of bone," *Medical Engineering and Physics,* vol. 20, no. 2, pp. 92-102, 1998.
[31] A. K. Nair, A. Gautieri, S.-W. Chang, and M. J. Buehler, "Molecular mechanics of mineralized collagen fibrils in bone," *Nature Communications,* vol. 4, pp. 1724-1724, 2013.
[32] A. J. Salgado, O. P. Coutinho, and R. L. Reis, "Bone tissue engineering: state of the art and future trends," (in eng), *Macromol Biosci,* vol. 4, no. 8, pp. 743-65, Aug 9 2004.
[33] N. Sims and J. Martin, "Coupling signals between the osteoclast and osteoblast: how are messages transmitted between these temporary visitors to the bone surface?," *Front. Endocrinol.,* 2015.
[34] H. Chen, T. Senda, and K. Kub, "The osteocyte plays multiple roles in bone remodeling and mineral homeostasis," *Med Mol Morphol,* 2015.
[35] T. Nakashima, "[Bone and Calcium Research Update 2015. Regulation of bone remodeling by osteocytes]," (in jpn), *Clin Calcium,* vol. 25, no. 1, pp. 21-8, Jan 2015.
[36] Ebah. (3 june). *Histologia - Tecido ósseo*. Available: http://www.ebah.com.br/content/ABAAAg7qsAI/histologia-tecido-osseo
[37] C. C. Verheyen, J. R. de Wijn, C. A. van Blitterswijk, and K. de Groot, "Evaluation of hydroxylapatite/poly(L-lactide) composites: mechanical behavior," (in eng), *J Biomed Mater Res,* vol. 26, no. 10, pp. 1277-96, Oct 1992.
[38] T. K.M., J. S.A., and C. A.R., *Veterinary surgery: small animal* (Fracture Biology and Biomechanics, no. 41). Elsivier, 2012.
[39] A. Ho-Shui-Ling, J. Bolander, L. E. Rustom, A. W. Johnson, F. P. Luyten, and C. Picart, "Bone regeneration strategies: Engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives," (in eng), *Biomaterials,* vol. 180, pp. 143-162, Oct 2018.
[40] M. Miyazaki, H. Tsumura, J. C. Wang, and A. Alanay, "An update on bone substitutes for spinal fusion," *European spine journal,* vol. 18, no. 6, pp. 783-799, 2009.

[41] J. V. Lobato *et al.*, "Titanium dental implants coated with Bonelike®: Clinical case report," *Thin Solid Films,* vol. 515, no. 1, pp. 279-284, 2006/09/25/ 2006.
[42] N. T., "Bone and Calcium Research Update 2015. Regulation of bone remodeling by osteocytes.," *Clin Calcium. ,* vol. 25, no. 1, pp. 21-28, 2015
[43] J. M. Kanczler and R. O. C. Oreffo, "Osteogenesis and Angiogenesis: The Potential for Engineering Bone," *European Cells and Materials,* vol. 15, pp. 100-114, 2008.
[44] C. L. VanPutte, J. L. Regan, and A. Russo, *Seeley's anatomy & physiology*. McGraw-Hill Education, 2017.
[45] M. Gutierres, M. A. Lopes, N. S. Hussain, A. T. Cabral, L. Almeida, and J. D. Santos, "Substitutos Ósseos : conceitos gerais e estado actual," *Arquivos de Medicina,* vol. 19, pp. 153-162, 2005.
[46] T. A. Einhorn and L. C. Gerstenfeld, "Fracture healing: mechanisms and interventions," *Nature Reviews Rheumatology,* vol. 11, no. 1, p. 45, 2015.
[47] M. Doblaré, J. M. García, and M. J. Gómez, "Modelling bone tissue fracture and healing: A review," *Engineering Fracture Mechanics,* vol. 71, no. 13-14, pp. 1809-1840, 2004.
[48] J. E. Davies and M. M. Hosseini, "Histodymanic of endosseous wound healing," J. E. Davies, Ed.: Academic Press, 2000.
[49] M. Braddock, P. Houston, C. Campbell, and P. Ashcroft, "Born again bone: tissue engineering for bone repair," *News in Physiological Sciences,* vol. 16, pp. 208-213, 2001.
[50] I. H. Kalfas, P. Houston, C. Campbell, and P. Ashcroft, "Principles of bone healing," *Neurosurg Focus,* vol. 10, no. 4, pp. 1-4, 2001.
[51] M. Gutierres *et al.*, "Histological and scanning electron microscopy analyses of bone/implant interface using the novel Bonelike synthetic bone graft," *Journal of Orthopaedic Research,* vol. 24, no. 5, pp. 953-958, 2006.
[52] C. Schlundt *et al.*, "Macrophages in bone fracture healing: Their essential role in endochondral ossification," *Bone,* 2015.
[53] M. D. DeLacure, "Physiology of bone healing and bone grafts," *Otolaryngologic Clinics of North America,* vol. 27, no. 5, pp. 859-874, 1994.
[54] J. Sela and I. Bab, "Healing of Bone Fracture: General Concepts," J. Sela and I. Bab, Eds.: Springer US, 2013, pp. 1-8.
[55] C. Sfeir, L. Ho, B. A. Doll, K. Azari, and J. O. Hollinger, "Fracture Repair," G. E. F. J.R. Lieberman, Ed.: Springer, 2005, pp. 21-44.
[56] M. J. Olszta *et al.*, "Bone structure and formation: A new perspective," *Materials Science and Engineering R: Reports,* vol. 58, no. 3-5, pp. 77-116, 2007.
[57] E. Kon *et al.*, "Platelet-rich plasma: intra-articular knee injections produced favorable results on degenerative cartilage lesions," (in English), *Knee Surgery, Sports Traumatology, Arthroscopy,* vol. 18, no. 4, pp. 472-479, 2010/04/01 2010.
[58] T. A. Einhorn and L. C. Gerstenfeld, "Fracture healing: mechanisms and interventions," *Nature Reviews Rheumatology,* vol. 11, no. 1, pp. 45-54, 2014.
[59] M. Melnyk, T. Henke, L. Claes, and P. Augat, "Revascularisation during fracture healing with soft tissue injury," *Archives of Orthopaedic and Trauma Surgery,* vol. 128, no. 10, pp. 1159-1165, 2008.
[60] J. H. Holstein *et al.*, "Endostatin Inhibits Callus Remodeling during Fracture Healing in Mice," *Journal of Orthopaedic Research,* vol. 31, no. 10, pp. 1579-1584, 2013.
[61] F. Chen and X. Liu, "Advancing biomaterials of human origin for tissue engineering," *Progress in Polymer Science,* vol. 53, pp. 86-168, 2016.
[62] S. V. Dorozhkin, "Calcium Orthophosphate-Based Bioceramics," *Materials,* vol. 6, no. 9, pp. 3840-3942, 09/06
06/20/received
08/07/revised
08/19/accepted 2013.
[63] D. Williams, *Revisiting the definition of biocompatibility*. 2003, pp. 10-3.

[64] D. F. Williams, "On the mechanisms of biocompatibility," *Biomaterials,* vol. 29, no. 20, pp. 2941-2953, 2008/07/01/ 2008.
[65] A. Srivastav, "An overview of metallic biomaterials for bone support and replacement," L. Anthony, Ed., 2011, pp. 153-168.
[66] A. Bhowmick, N. Pramanik, and P. P Kundu, *Development of bone-like zirconium oxide nanoceramic modified chitosan based porous nanocomposites for biomedical application*. 2016.
[67] D. J. Hall, T. M. Turner, and R. M. Urban, "Healing bone lesion defects using injectable CaSO4/CaPO4-TCP bone graft substitute compared to cancellous allograft bone chips in a canine model," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 0, no. 0, 2018.
[68] S. H. Teoh, "Introduction to biomaterials engineering and processing – an overview. ," in *Engineering Materials for Biomedical Applications*, vol. Volume 1(Biomaterials Engineering and Processing Series, no. Volume 1): WORLD SCIENTIFIC, 2004, pp. 1-1-1-16.
[69] P. V. Giannoudis, H. Dinopoulos, and E. Tsiridis, "Bone substitutes: an update," (in eng), *Injury,* vol. 36 Suppl 3, pp. S20-7, Nov 2005.
[70] M. Gutierres *et al.*, "Biological Behaviour of Bonelike® Graft Implanted in the Tibia of Humans," *Key Engineering Materials,* vol. 284-286, pp. 1041-1044, 2005.
[71] T. W. Bauer and G. F. Muschler, "Bone graft materials. An overview of the basic science," *Clinical Orthopaedics & Related Research,* no. 371, pp. 10-27, 2000.
[72] R. LeGeros, S. Lin, R. Rohanizadeh, D. Mijares, and J. LeGeros, "Biphasic calcium phosphate bioceramics: preparation, properties and applications," *Journal of materials science: Materials in Medicine,* vol. 14, no. 3, pp. 201-209, 2003.
[73] L. M. Atayde, P. P. Cortez, A. Afonso, M. Santos, A. C. Maurício, and J. D. Santos, "Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike®) on osteoregeneration," *Journal of Biomedical Materials Research - Part B Applied Biomaterials,* vol. 103, no. 2, pp. 292-304, 2015.
[74] B. S.M., P. A.E., T. E.S., and H. J., "Bioceramics: Past, present and for the future," *Journal of the European Ceramic Society,* vol. 28, pp. 1319–1327, 2008.
[75] H. R. Ramay and M. Zhang, "Preparation of porous hydroxyapatite scaffolds by combination of the gel-casting and polymer sponge method," *Biomaterials,* vol. , no. 24, pp. 3293–3302, 2003
[76] J. J. Li *et al.*, "Efficacy of novel synthetic bone substitutes in the reconstruction of large segmental bone defects in sheep tibiae.," *Biomed. Mater. 11* 2016.
[77] S. Shanbhag, N. Pandis, K. Mustafa, J. R. Nyengaard, and A. Stavropoulos, "Bone tissue engineering in oral peri-implant defects in preclinical in vivo research: a systematic review and meta-analysis," *J Tissue Eng Regen Med. ,* vol. 12, no. 1, pp. e336-e349, 2017. Epub 2017 May 31
[78] S. J. Bryant, J. L. Cuy, K. D. Hauch, and B. D. Ratner, "Photo-patterning of porous hydrogels for tissue engineering," *Biomaterials,* vol. 28, no. 19, pp. 2978-2986, 2007.
[79] M. H. P. Silva, A. F. Lemos, J. M. F. Ferreira, and J. D. Santos, "Mechanical Characterisation of Porous Glass Reinforced Hydroxyapatite Ceramics: Bonelike®," *Materials Research,* vol. 6, no. 3, pp. 321-325, 2003.
[80] R. B.D., H. A.S., and S. F. J., *Biomaterial Science* (Biomaterials Science: A Multidisciplinary Endeavor). 2004, pp. 1-9.
[81] B. Ratner, A. Hoffman, F. Schoen, and J. Lemons, *Biomaterials Science*. 1996.
[82] L. M. Atayde *et al.*, "A new sheep model with automatized analysis of biomaterial-induced bone tissue regeneration," *Journal of Materials Science: Materials in Medicine,* vol. 25, no. 8, pp. 1885-901, 2014.
[83] J. J. Li *et al.*, "Effects of Material–Tissue Interactions on Bone Regeneration Outcomes Using Baghdadite Implants in a Large Animal Model," *Advanced Healthcare Materials* 2018.
[84] W. Wang and K. W. Yeung, "Bone grafts and biomaterials substitutes for bone defect repair: A review," *Bioactive Materials,* 2017.

[85] L. Atayde, "Substitutos Ósseos Para Regeneração do Tecido Ósseo: Estudos In Vivo e Futuras Aplicações Clínicas Em Medicina Veterinária," 2014.
[86] P. HC, E. A, and K. P, "Autologous Bone Graft: Properties and Techniques," *J Orthop Trauma.,* vol. 1, no. 24, pp. 36-40, 2010 Mar.
[87] T. Guda, C. Labella, R. Chan, and R. Hale, "Quality of bone healing: Perspectives and assessment techniques," *Wound Repair and Regeneration,* vol. 22, no. S1, pp. 39-49, 2014.
[88] T. G. C. L. R. C. R. Hale, "Quality of bone healing: Perspectives and assessment techniques," *Wound Rep Reg,* vol. 22, pp. 39-49, 2014.
[89] S.-Y. Park, K.-H. Kim, S.-Y. Shin, K.-T. Koo, Y.-M. Lee, and Y.-J. Seol, "Dual delivery of rhPDGF-BB and bone marrow mesenchymal stromal cells expressing the BMP2 gene enhance bone formation in a critical-sized defect model," *Tissue Engineering Part A,* vol. 19, no. 21-22, pp. 2495-2505, 2013.
[90] M. Kamitakahara, C. Ohtsuki, and T. Miyazaki, "Behavior of ceramic biomaterials derived from tricalcium phosphate in physiological condition," *Journal of biomaterials applications,* vol. 23, no. 3, pp. 197-212, 2008.
[91] C. P. Atayde LM, Pereira T, Armada-da-Silva P, Afonso A, Lopes MA, Santos JD, Maurício AC., "A new sheep model with computing automatized analysis to evaluate the in vivo biomaterials behavior on bone tissue regeneration.," *Journal of Materials Science: Materials in Medicine,* 2014.
[92] A. Maiors, C. Boehm, H. Nitto, R. Midura, and G. Muschler, "Characterization Of Human Bone Marrow Stromal Cells With Respect To Osteoblastic Differentiation," *Journal of Bone and Mineral Research,* vol. 11, p. S259, 1996.
[93] H. Burchardt, "Biology of bone transplantation," *The Orthopedic Clinics of North America,* vol. 18, no. 2, pp. 187-196, 1987.
[94] S. M. Day, R. F. Ostrum, E. Y. Chao, C. T. Rubin, H. T. Aro, and T. A. Einhorn, "Bone injury, regeneration, and repair," *Orthopaedic basic science: biology and biomechanics of the musculoskeletal system. 2nd ed. Rosemont (IL): American Academy of Orthopaedic Surgeons,* pp. 371-99, 2000.
[95] E. Erbe, J. Marx, T. Clineff, and L. Bellincampi, "Potential of an ultraporous β-tricalcium phosphate synthetic cancellous bone void filler and bone marrow aspirate composite graft," *European Spine Journal,* vol. 10, no. 2, pp. S141-S146, 2001.
[96] P. V. Giannoudis, H. Dinopoulos, and E. Tsiridis, "Bone substitutes: an update," *Injury,* vol. 36, no. 3, pp. S20-S27, 2005.
[97] G. Arthurs, G. T.J. and D. N. C., Eds. *Bone grafts and alternatives*, BSAVA Manual of canine and feline repair and management ed. 2016.
[98] D. Tang, R. S. Tare, L.-Y. Yang, D. F. Williams, K.-L. Ou, and R. O. Oreffo, "Biofabrication of bone tissue: approaches, challenges and translation for bone regeneration," *Biomaterials,* vol. 83, pp. 363-382, 2016.
[99] T. Balaguer *et al.*, "Combination of blood and biphasic calcium phosphate microparticles for the reconstruction of large bone defects in dog: A pilot study," *Journal of Biomedical Materials Research Part A,* vol. 106, no. 7, pp. 1842-1850, 2018.
[100] J. M. Bouler, P. Pilet, O. Gauthier, and E. Verron, "Biphasic calcium phosphate ceramics for bone reconstruction: A review of biological response," (in eng), *Acta Biomater,* vol. 53, pp. 1-12, Apr 15 2017.
[101] T. Kurien, R. Pearson, and B. Scammell, "Bone graft substitutes currently available in orthopaedic practice: the evidence for their use," *Bone Joint J,* vol. 95, no. 5, pp. 583-597, 2013.
[102] H. Ueno *et al.*, "Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs," *Biomaterials,* vol. 20, no. 15, pp. 1407-1414, 1999.
[103] C. G. Finkemeier, "Bone-grafting and bone-graft substitutes," *JBJS,* vol. 84, no. 3, pp. 454-464, 2002.

[104] G. Schmidmaier *et al.*, "Quantitative assessment of growth factors in reaming aspirate, iliac crest, and platelet preparation," *Bone,* vol. 39, no. 5, pp. 1156-1163, 2006.
[105] S. N. Khan, F. P. Cammisa Jr, H. S. Sandhu, A. D. Diwan, F. P. Girardi, and J. M. Lane, "The biology of bone grafting," *JAAOS-Journal of the American Academy of Orthopaedic Surgeons,* vol. 13, no. 1, pp. 77-86, 2005.
[106] R. Boudrieau, A. Tidwell, S. Ullman, and B. Gores, "Correction of mandibular nonunion and malocclusion by plate fixation and autogenous cortical bone grafts in two dogs," *Journal of the American Veterinary Medical Association,* vol. 204, no. 5, pp. 744-750, 1994.
[107] B. Séguin, P. J. Walsh, D. R. Mason, E. R. Wisner, J. L. Parmenter, and W. S. Dernell, "Use of An ipsilateral Vascularized Ulnar Transposition Autograft for Limb-Sparing Surgery of the Distal Radius in Dogs: An Anatomic and Clinical Study," *Veterinary Surgery,* vol. 32, no. 1, pp. 69-79, 2003.
[108] J. D. Bacher and R. E. Schmidt, "Effects of autogenous cancellous bone on healing of homogenous cortical bone grafts," (in eng), *J Small Anim Pract,* vol. 21, no. 4, pp. 235-45, Apr 1980.
[109] B. A. Ashton, T. D. Allen, C. Howlett, C. Eaglesom, A. Hattori, and M. Owen, "Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo," *Clinical orthopaedics and related research,* no. 151, pp. 294-307, 1980.
[110] S. e. al., "Use of an Ipsilateral Vascularized Ulnar Transposition Autograft for Limb-Sparing Surgery of the Distal Radius in Dogs: An Anatomic and Clinical Study," pp. 69-79, 2003.
[111] J. Deleu and J. Trueta, "Vascularisation of bone grafts in the anterior chamber of the eye," *Bone & Joint Journal,* vol. 47, no. 2, pp. 319-329, 1965.
[112] W. F. Enneking, H. Burchardt, J. J. Puhl, and G. Piotrowski, "Physical and biological aspects of repair in dog cortical-bone transplants," *The Journal of bone and joint surgery. American volume,* vol. 57, no. 2, pp. 237-252, 1975.
[113] C. R. Perry, "Bone repair techniques, bone graft, and bone graft substitutes," *Clinical Orthopaedics and Related Research®,* vol. 360, pp. 71-86, 1999.
[114] R. Zamborsky, A. Svec, M. Bohac, M. Kilian, and M. Kokavec, "Infection in Bone Allograft Transplants," *Exp Clin Transplant,* vol. 14, pp. 484-90, 2016.
[115] A. LM., "Substitutos Ósseos Para Regeneração do Tecido Ósseo: Estudos In Vivo e Futuras Aplicações Clínicas Em Medicina Veterinária.," *Porto: ICBAS - Universidade do Porto,* 2014.
[116] P. P. Cortez *et al.*, "A glass-reinforced hydroxyapatite and surgical-grade calcium sulfate for bone regeneration : In vivo biological behavior in a sheep model," *Journal of Biomaterials Applications,* vol. 27, no. 2, pp. 201-217, 2011.
[117] R. LeGeros *et al.*, "Significance of the Porosity and Physical Chemistry of Calcium Phosphate Ceramics Biodegradation-Bioresorption," *Annals of the New York Academy of Sciences,* vol. 523, no. 1, pp. 268-271, 1988.
[118] J. Lobato *et al.*, "Assessment of the potential of Bonelike® graft for bone regeneration by using an animal model," in *Key Engineering Materials*, 2005, vol. 284, pp. 877-880: Trans Tech Publ.
[119] A. Dupraz, J. Delecrin, A. Moreau, P. Pilet, and N. Passuti, "Long-term bone response to particulate injectable ceramic," *Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and the Australian Society for Biomaterials,* vol. 42, no. 3, pp. 368-375, 1998.
[120] V. Karageorgiou and D. Kaplan, "Porosity of 3D biomaterial scaffolds and osteogenesis," *Biomaterials,* vol. 26, no. 27, pp. 5474-5491, 2005.
[121] S. Marcelo Henrique Prado da, L. Alexandra Fernandes, F. José Maria da Fonte, and S. José Domingos, *Mechanical characterisation of porous glass reinforced hydroxyapatite ceramics: Bonelike®*. 2003.
[122] E. S. Sanzana, M. Navarro, M. P. Ginebra, J. A. Planell, A. C. Ojeda, and H. A. Montecinos, "Role of porosity and pore architecture in the in vivo bone regeneration capacity of biodegradable

glass scaffolds," *Journal of Biomedical Materials Research Part A,* vol. 102, no. 6, pp. 1767-1773, 2014.
[123] K. A. Hing, "Bioceramic bone graft substitutes: influence of porosity and chemistry," *International journal of applied ceramic technology,* vol. 2, no. 3, pp. 184-199, 2005.
[124] H. Götz, M. Müller, A. Emmel, U. Holzwarth, R. Erben, and R. Stangl, "Effect of surface finish on the osseointegration of laser-treated titanium alloy implants," *Biomaterials,* vol. 25, no. 18, pp. 4057-4064, 2004.
[125] J. Le Huec, D. Clement, E. Lesprit, and J. Faber, "The use of calcium phosphates, their biological properties," *European Journal of Orthopaedic Surgery & Traumatology,* vol. 10, no. 4, pp. 223-229, 2000.
[126] R. Cancedda, P. Giannoni, and M. Mastrogiacomo, "A tissue engineering approach to bone repair in large animal models and in clinical practice," (in eng), *Biomaterials,* vol. 28, no. 29, pp. 4240-50, Oct 2007.
[127] F. Hao, L. Qin, J. Liu, J. Chang, Z. Huan, and L. Wu, "Assessment of calcium sulfate hemihydrate-Tricalcium silicate composite for bone healing in a rabbit femoral condyle model," (in eng), *Mater Sci Eng C Mater Biol Appl,* vol. 88, pp. 53-60, Jul 1 2018.
[128] Z. J. Yang, H. Yuan, P. Zou, W. Tong, S. Qu, and X. D. Zhang, "Osteogenic responses to extraskeletally implanted synthetic porous calcium phosphate ceramics: an early stage histomorphological study in dogs," *Journal of Materials Science: Materials in Medicine,* vol. 8, no. 11, pp. 697-701, 1997.
[129] T. Kokubo, *Bioceramics and Their Clinical Applications*. 2007.
[130] W. R. Moore, S. E. Graves, and G. I. Bain, "Synthetic bone graft substitutes," *ANZ journal of surgery,* vol. 71, no. 6, pp. 354-361, 2001.
[131] R. J. Miron *et al.*, "Osteoinductive potential of 4 commonly employed bone grafts," *Clinical oral investigations,* vol. 20, no. 8, pp. 2259-2265, 2016.
[132] P. Pinto *et al.*, "Therapeutic Strategies for Bone Regeneration: The Importance of Biomaterials Testing in Adequate Animal Models," *Advanced Composite Materials,* pp. 275-319, 2016.
[133] J. Lobato *et al.*, "Assessment of Bonelike® graft with a resorbable matrix using an animal model," *Thin solid films,* vol. 515, no. 1, pp. 362-367, 2006.
[134] K. L. Low, S. H. Tan, S. H. S. Zein, J. A. Roether, V. Mouriño, and A. R. Boccaccini, "Calcium phosphate-based composites as injectable bone substitute materials," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 94, no. 1, pp. 273-286, 2010.
[135] M. Kamitakahara, C. Ohtsuki, and T. Miyazaki, *Review Paper: Behavior of Ceramic Biomaterials Derived from Tricalcium Phosphate in Physiological Condition*. 2008, pp. 197-212.
[136] P. C. Salgado *et al.*, "Bone remodeling, biomaterials and technological applications: revisiting basic concepts," *Journal of Biomaterials and Nanobiotechnology,* vol. 2, no. 03, p. 318, 2011.
[137] K. Mahan and M. Carey, "Hydroxyapatite as a bone substitute," *Journal of the American Podiatric Medical Association,* vol. 89, no. 8, pp. 392-397, 1999.
[138] L. AS, *"Arthrodesis" In: Slater D. Textbook of small animal surgery*, 3 ed. 2002.
[139] J. M. Lane and S. N. Khan, "Bone grafts of the 20th century: multiple purposes, materials and goals," *Orthopedics Today,* 2000.
[140] N. Scarborough, E. White, J. Hughes, A. Manrique, and J. Poser, "Allograft safety: viral inactivation with bone demineralization," *Contemporary orthopaedics,* vol. 31, no. 4, pp. 257-261, 1995.
[141] T. Boyce, J. Edwards, and N. Scarborough, "Allograft bone: the influence of processing on safety and performance," *Orthopedic Clinics,* vol. 30, no. 4, pp. 571-581, 1999.
[142] P. R. Klokkevold and S. A. Jovanovic, "Advanced implant surgery and bone grafting techniques," *Carranza's Clinical Periodontology, 9th Edition. Philadelphia: WB Saunders Co,* pp. 907-908, 2002.
[143] L. Hench, "Molecular design of bioactive glasses and ceramics for implants," *Ceramics: Towards the 21st Century Ceram Soc of Japan,* pp. 519-34, 1991.

[144] M. Costa, M. Gutierres, L. Almeida, M. Lopes, J. D. Santos, and M. H. F. Fernandes, "In vitro mineralisation of human bone marrow cells cultured on Bonelike®," in *Key Engineering Materials*, 2004, vol. 254, pp. 821-824: Trans Tech Publ.

[145] J. Fischer, A. Kolk, C. Pautke, P. Warnke, C. Plank, and R. Smeets, "Future of local bone regeneration–protein versus gene therapy," *Journal of cranio-maxillo-facial surgery,* vol. 39, no. 1, pp. 54-64, 2011.

[146] N. Bormann, P. Schwabe, M. Smith, and B. Wildemann, "Analysis of parameters influencing the release of antibiotics mixed with bone grafting material using a reliable mixing procedure," *Bone,* vol. 59, pp. 162-172, 2014.

[147] M. Bohner, L. Galea, and N. Doebelin, "Calcium phosphate bone graft substitutes: Failures and hopes," *Journal of the European Ceramic Society,* vol. 32, no. 11, pp. 2663-2671, 2012.

[148] P. Parida, A. Behera, and S. C. Mishra, "Classification of Biomaterials used in Medicine," *International Journal of Advances in Applied Sciences,* vol. 1, no. 3, pp. 125-129, 2012.

[149] H. Leda, "Engineering materials for biomedical applications," *Publisher University of Technology Poznan,* 2011.

[150] E. Panzeri *et al.*, "Chromosomal aberrations in bladder cancer: fresh versus formalin fixed paraffin embedded tissue and targeted FISH versus wide microarray-based CGH analysis," *PLoS One,* vol. 6, no. 9, p. e24237, 2011.

[151] S. V. Dorozhkin, "Bioceramics of calcium orthophosphates," *Biomaterials,* vol. 31, no. 7, pp. 1465-1485, 2010.

[152] C. Yang, O. Unursaikhan, J. S. Lee, U. W. Jung, C. S. Kim, and S. H. Choi, "Osteoconductivity and biodegradation of synthetic bone substitutes with different tricalcium phosphate contents in rabbits," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 102, no. 1, pp. 80-88, 2014.

[153] C. Knabe *et al.*, "Effect of silicon-doped calcium phosphate bone grafting materials on bone regeneration and osteogenic marker expression after implantation in the ovine scapula," (in eng), *J Biomed Mater Res B Appl Biomater,* May 16 2018.

[154] M. Hamadouche and L. Sedel, "Ceramics in orthopaedics," *Bone & Joint Journal,* vol. 82, no. 8, pp. 1095-1099, 2000.

[155] P. P. Cortez *et al.*, "The in vivo performance of an alkali-free bioactive glass for bone grafting, FastOs((R)) BG, assessed with an ovine model," (in eng), *J Biomed Mater Res B Appl Biomater,* vol. 105, no. 1, pp. 30-38, Jan 2017.

[156] M. Bohner, S. Tadier, N. van Garderen, A. de Gasparo, N. Döbelin, and G. Baroud, "Synthesis of spherical calcium phosphate particles for dental and orthopedic applications," *Biomatter,* vol. 3, no. 2, p. e25103, 2013.

[157] D. P. Lund *et al.*, "Liver transplantation in newborn liver failure: treatment for neonatal hemochromatosis," *Transplant Proc,* vol. 25, no. 1 Pt 2, pp. 1068-71, Feb 1993.

[158] L. L. Hench, *An Introduction to Bioceramics*, 2 ed. Florida, 2006.

[159] R. Sousa *et al.*, "A clinical report of bone regeneration in maxillofacial surgery using Bonelike® synthetic bone graft," *Journal of biomaterials applications,* vol. 22, no. 4, pp. 373-385, 2008.

[160] M. J. Jackson and W. Ahmed, *Surface engineered surgical tools and medical devices*. Springer Science & Business Media, 2007.

[161] B. Kanter, A. Vikman, T. Bruckner, M. Schamel, U. Gbureck, and A. Ignatius, "Bone regeneration capacity of magnesium phosphate cements in a large animal model," (in eng), *Acta Biomater,* vol. 69, pp. 352-361, Mar 15 2018.

[162] A. D. Talley, M. A. McEnery, K. N. Kalpakci, K. J. Zienkiewicz, D. A. Shimko, and S. A. Guelcher, "Remodeling of injectable, low-viscosity polymer/ceramic bone grafts in a sheep femoral defect model," (in eng), *J Biomed Mater Res B Appl Biomater,* vol. 105, no. 8, pp. 2333-2343, Nov 2017.

[163] A. D. Talley, L. A. Boller, K. N. Kalpakci, D. A. Shimko, D. L. Cochran, and S. A. Guelcher, "Injectable, compression-resistant polymer/ceramic composite bone grafts promote lateral

ridge augmentation without protective mesh in a canine model," (in eng), *Clin Oral Implants Res,* vol. 29, no. 6, pp. 592-602, Jun 2018.
[164] S. V. Dorozhkin, "Amorphous calcium orthophosphates: nature, chemistry and biomedical applications," *Int. J. Mater. Chem,* vol. 2, no. 1, pp. 19-46, 2012.
[165] R. Duan, D. Barbieri, F. de Groot, J. D. de Bruijn, and H. Yuan, "Modulating Bone Regeneration in Rabbit Condyle Defects with Three Surface-Structured Tricalcium Phosphate Ceramics," (in eng), *ACS Biomater Sci Eng,* vol. 4, no. 9, pp. 3347-3355, Sep 10 2018.
[166] O. Gauthier *et al.*, "In vivo bone regeneration with injectable calcium phosphate biomaterial: a three-dimensional micro-computed tomographic, biomechanical and SEM study," (in eng), *Biomaterials,* vol. 26, no. 27, pp. 5444-53, Sep 2005.
[167] A. Wildburger *et al.*, "Sinus Floor Augmentation Comparing an In Situ Hardening Biphasic Calcium Phosphate (Hydroxyapatite/beta-Tricalcium Phosphate) Bone Graft Substitute with a Particulate Biphasic Calcium Phosphate (Hydroxyapatite/beta-Tricalcium Phosphate) Bone Graft Substitute: An Experimental Study in Sheep," (in eng), *Tissue Eng Part C Methods,* vol. 23, no. 7, pp. 404-411, Jul 2017.
[168] C. Yang, O. Unursaikhan, L. Jung Seok, U. Jung, C. Kim, and S. Choi, *Osteoconductivity and biodegradation of synthetic bone substitutes with different tricalcium phosphate contents in rabbits*. 2014, pp. 80-88.
[169] C. M. Kelly, R. M. Wilkins, S. Gitelis, C. Hartjen, J. T. Watson, and P. T. Kim, "The use of a surgical grade calcium sulfate as a bone graft substitute: results of a multicenter trial," *Clinical Orthopaedics and Related Research®,* vol. 382, pp. 42-50, 2001.
[170] C. Laurencin, Y. Khan, and S. F. El-Amin, "Bone graft substitutes," *Expert review of medical devices,* vol. 3, no. 1, pp. 49-57, 2006.
[171] D. I. Ilan and A. L. Ladd, "Bone graft substitutes," *Operative Techniques in Plastic and Reconstructive Surgery,* vol. 9, no. 4, pp. 151-160, 2002.
[172] J. E. Fleming, C. N. Cornell, and G. F. Muschler, "Bone cells and matrices in orthopedic tissue engineering," *Orthopedic Clinics,* vol. 31, no. 3, pp. 357-374, 2000.
[173] S. Stevenson, "Biology of bone grafts," *Orthopedic Clinics,* vol. 30, no. 4, pp. 543-552, 1999.
[174] K. J. Burg, S. Porter, and J. F. Kellam, "Biomaterial developments for bone tissue engineering," *Biomaterials,* vol. 21, no. 23, pp. 2347-2359, 2000.
[175] J. Keating and M. McQueen, "Substitutes for autologous bone graft in orthopaedic trauma," *Bone & Joint Journal,* vol. 83, no. 1, pp. 3-8, 2001.
[176] E. Damien and P. Revell, "Coralline hydroxyapatite bone graft substitute: a review of experimental studies and biomedical applications," *Journal of Applied Biomaterials and Biomechanics,* vol. 2, no. 2, pp. 65-73, 2004.
[177] D. Le Nihouannen *et al.*, "Ectopic bone formation by microporous calcium phosphate ceramic particles in sheep muscles," (in eng), *Bone,* vol. 36, no. 6, pp. 1086-93, Jun 2005.
[178] C. J. Damien and J. R. Parsons, "Bone graft and bone graft substitutes: a review of current technology and applications," (in eng), *J Appl Biomater,* vol. 2, no. 3, pp. 187-208, Fall 1991.
[179] S. Lu *et al.*, "Settable polymer/ceramic composite bone grafts stabilize weight-bearing tibial plateau slot defects and integrate with host bone in an ovine model," (in eng), *Biomaterials,* vol. 179, pp. 29-45, Oct 2018.
[180] D. J. Hak, "The use of osteoconductive bone graft substitutes in orthopaedic trauma," *Journal of the American Academy of Orthopaedic Surgeons,* vol. 15, no. 9, pp. 525-536, 2007.
[181] I. R. Dias *et al.*, "Preclinical and Translational Studies in Small Ruminants (Sheep and Goat) as Models for Osteoporosis Research," (in eng), *Curr Osteoporos Rep,* vol. 16, no. 2, pp. 182-197, Apr 2018.
[182] E. B. Nery, R. Z. Legeros, K. L. Lynch, and K. Lee, "Tissue response to biphasic calcium phosphate ceramic with different ratios of HA/βTCP in periodontal osseous defects," *Journal of periodontology,* vol. 63, no. 9, pp. 729-735, 1992.

[183] M. N. Rahaman *et al.*, "Bioactive glass in tissue engineering," *Acta biomaterialia,* vol. 7, no. 6, pp. 2355-2373, 2011.
[184] L. J. Jha, J. D. Santos, and J. C. Knowles, "Characterization of apatite layer formation on P2O5-CaO, P2O5-CaO-Na2O, and P2O5-CaO-Na2O-Al2O3 glass hydroxyapatite composites," *Journal of Biomedical Materials Research - Part A,* vol. 31, no. 4, pp. 481-486, 1996.
[185] J. D. Santos, L. J. Jha, and F. J. Monteiro, "In vitro calcium phosphate formation on SiO2-Na2O-CaO-P2O5 glass reinforced hydroxyapatite composite: A study by XPS analysis," *Journal of Materials Science: Materials in Medicine,* vol. 7, no. 3, pp. 181-185, 1996.
[186] J. Santos, G. Hastings, and J. Knowles, "Sintered hydroxyapatite compositions and method for the preparation thereof," *Worldwide Application (PCT), Patent,* no. 1, p. 189, 1999.
[187] Biosckin. BL [Online].
[188] J. Santos, L. Jha, and F. Monteiro, "In vitro calcium phosphate formation on SiO 2-Na 2 O-CaO-P 2 O 5 glass reinforced hydroxyapatite composite: A study by XPS analysis," *Journal of Materials Science: Materials in Medicine,* vol. 7, no. 3, pp. 181-185, 1996.
[189] J. C. Knowles, G. W. Hastings, and J. D. Santos, "Sintered hydroxyapatite compositions and method for the preparation thereof," 2000.
[190] J. Knowles, G. Hastings, and J. Santos, "Sintered hydroxyapatite compositions and method for the preparation thereof," *Great Britain patent WO/2000/068164,* vol. 16, p. 2000, 2000.
[191] G. Georgiou and J. C. Knowles, "Glass reinforced hydroxyapatite for hard tissue surgery--part 1: Mechanical properties," (in eng), *Biomaterials,* vol. 22, no. 20, pp. 2811-5, Oct 2001.
[192] T. Uchino, K. Yamaguchi, I. Suzuki, M. Kamitakahara, M. Otsuka, and C. Ohtsuki, "Hydroxyapatite formation on porous ceramics of alpha-tricalcium phosphate in a simulated body fluid," (in eng), *J Mater Sci Mater Med,* vol. 21, no. 6, pp. 1921-6, Jun 2010.
[193] J. Campos *et al.*, "Dental Pulp Stem Cells and Bonelike® for Bone Regeneration in Ovine Model," *Regenerative Biomaterial* 2018.
[194] P. P. Cortez *et al.*, "Characterization and preliminary in vivo evaluation of a novel modified hydroxyapatite produced by extrusion and spheronization techniques," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 99, no. 1, pp. 170-179, 2011.
[195] M. Lopes, J. D Santos, F. Monteiro, and J. Knowles, *Glass-reinforced hydroxyapatite: A comprehensive study of the effect of glass composition on the crystallography of the composite*. 1998, pp. 244-251.
[196] Y. Shirosaki, C. M. Botelho, M. A. Lopes, and J. D. Santos, "Synthesis and characterization of chitosan-silicate hydrogel as resorbable vehicle for Bonelike® bone graft," *Journal of nanoscience and nanotechnology,* vol. 9, no. 6, pp. 3714-3719, 2009.
[197] C. JM *et al.*, "Application of Bonelike® as synthetic bone graft in orthopaedic and oral surgery in veterinary clinical cases," *Journal Biomaterials Research,* 2018.
[198] N. S. Cunningham, V. Paralkar, and A. H. Reddi, "Osteogenin and recombinant bone morphogenetic protein 2B are chemotactic for human monocytes and stimulate transforming growth factor beta 1 mRNA expression," (in eng), *Proc Natl Acad Sci U S A,* vol. 89, no. 24, pp. 11740-4, Dec 15 1992.
[199] W. I. Abdel-Fattah, S. H. E. Ashry, G. W. Ali, M. A. A. Hamid, A. G. El-Din, and B. El-Ashry, "Regeneration of periapical lesions post-endodontic treatment and periapical surgeries in experimental animals utilizing thermoresponsive nano-β-tricalcium phosphate/chitosan hydrogel: a proof of concept," *Biomedical Materials* vol. 12, no. 4, 2017.
[200] M. A. Lopes *et al.*, "Push-out testing and histological evaluation of glass reinforced hydroxyapatite composites implanted in the tibia of rabbits," *Journal of Biomedical Materials Research,* vol. 54, no. 4, pp. 463-469, 2001.
[201] M. Gutierres *et al.*, "Bone ingrowth in macroporous Bonelike® for orthopaedic applications," *Acta Biomaterialia,* vol. 4, no. 2, pp. 370-377, 2008/03/01/ 2008.
[202] S. Simske, R. Ayers, and T. Bateman, *Porous Materials for Bone Engineering*. 1997, pp. 151-182.

[203] A. Piattelli, A. Scarano, and C. Mangano, "Clinical and histologic aspects of biphasic calcium phosphate ceramic (BCP) used in connection with implant placement," *Biomaterials,* vol. 17, no. 18, pp. 1767-1770, 1996/09/01/ 1996.
[204] A. Argast and C. F. Tennis, "A Web Resource for the Study of Alkali Feldspars and Perthitic Textures Using Light Microscopy, Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy," *Journal of Geoscience Education,* vol. 52, no. 3, pp. 213-217, 2004.
[205] R. Bean, "Using the Scanning Electron Microscope for Discovery Based Learning in Undergraduate Courses," *Journal of Geoscience Education,* vol. 52, no. 3, pp. 250-253, 2004.
[206] J. Goldstein *et al.*, *Scanning Electron Microscopy and X-ray Microanalysis: Third Edition*, 3 ed. Springer US, 2003.
[207] J. P. Vacanti, M. A. Morse, W. M. Saltzman, A. J. Domb, A. Perez-Atayde, and R. Langer, "Selective cell transplantation using bioabsorbable artificial polymers as matrices," (in eng), *J Pediatr Surg,* vol. 23, no. 1 Pt 2, pp. 3-9, Jan 1988.
[208] R. Langer and J. Vacanti, "Advances in tissue engineering," (in eng), *J Pediatr Surg,* vol. 51, no. 1, pp. 8-12, Jan 2016.
[209] M. Dominici *et al.*, "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement," *Cytotherapy,* vol. 8, no. 4, pp. 315-7, 2006.
[210] G. F. Muschler, C. Nakamoto, and L. G. Griffith, "Engineering Principles of Clinical Cell-Based Tissue Engineering," *JBJS,* vol. 86, no. 7, pp. 1541-1558, 2004.
[211] K. J. B. C. A. M. H. Agis, "Dental pulp regeneration
In vitro and in vivo approaches," *J. Stomat. Occ. Med,* 2015.
[212] A. Graziano, R. d'Aquino, G. Laino, and G. Papaccio, "Dental pulp stem cells: a promising tool for bone regeneration," (in eng), *Stem Cell Rev,* vol. 4, no. 1, pp. 21-6, Spring 2008.
[213] P. D. Potdar and Y. D. Jethmalani, "Human dental pulp stem cells: Applications in future regenerative medicine," (in eng), *World J Stem Cells,* vol. 7, no. 5, pp. 839-51, Jun 26 2015.
[214] F. F. Demarco, M. C. Conde, B. N. Cavalcanti, L. Casagrande, V. T. Sakai, and J. E. Nor, "Dental pulp tissue engineering," (in eng), *Braz Dent J,* vol. 22, no. 1, pp. 3-13, 2011.
[215] S. Gronthos, M. Mankani, J. Brahim, P. G. Robey, and S. Shi, "Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo," *Proc Natl Acad Sci U S A,* vol. 97, no. 25, pp. 13625-30, Dec 5 2000.
[216] D. L. Alge *et al.*, "Donor-matched comparison of dental pulp stem cells and bone marrow-derived mesenchymal stem cells in a rat model," *J Tissue Eng Regen Med,* vol. 4, no. 1, pp. 73-81, Jan 2010.
[217] T. P. A R Caseiro, J Bártolo, J D Santos, A L Luís, A C Maurício, "Mesenchymal Stem Cells and Biomaterials Systems – Perspectives for Skeletal Muscle Tissue Repair and Regeneration," *Procedia Engineering,* vol. 110, pp. 90-97, 2015.
[218] S. Gronthos *et al.*, "Stem cell properties of human dental pulp stem cells," *Journal of dental research,* vol. 81, no. 8, pp. 531-535, 2002.
[219] N. K. Y. Isobe, K. Nakao, K. Osawa, M. Ikeno, S. Yamanaka, Y. Okubo, K. Fujimura, K. Bessho, "Comparison of human mesenchymal stem cells derived from bone marrow, synovial fluid, adult dental pulp, and exfoliated deciduous tooth pulp," *Oral&Maxillo Facial Surgery,* journal 2015.
[220] D. M. Choumerianou, H. Dimitriou, and M. Kalmanti, "Stem cells: promises versus limitations," (in eng), *Tissue Eng Part B Rev,* vol. 14, no. 1, pp. 53-60, Mar 2008.
[221] C. J. Assis AC1, Jacoby BA, Ferreira RL, Castanheira P, Diniz SO, Cardoso VN, Goes AM, Ferreira AJ., "Time-dependent migration of systemically delivered bone marrow mesenchymal stem cells to the infarcted heart.," *Cell Transplantation,* vol. 19, no. 2, pp. 219-300, 2010.
[222] N. Askari, M. Yaghoobi, M. Shamsara, and S. Esmaeili-Mahani, "Tetracycline-regulated expression of OLIG2 gene in human dental pulp stem cells lead to mouse sciatic nerve regeneration upon transplantation," *Neuroscience,* vol. 305, pp. 197-208, 2015.

[223] A. Morsy *et al.*, *The potential of nanoparticles in stem cell differentiation and further therapeutic applications*. 2016, pp. 1550-1560.
[224] A. R. Caseiro, T. Pereira, P. J. Bártolo, J. D. Santos, A. L. Luís, and A. C. Maurício, InTech, Ed. *Chapter - Trends in Mesenchymal Stem Cells Applications for Skeletal Muscle Repair and Regeneration, in PROGRESS IN STEM CELL TRANSPLANTATION*. T Demirer 2015.
[225] A. R. Caseiro *et al.*, "The Potential Clinical Application of Mesenchymal Stem Cells from the Dental Pulp (DPSCs) for Bone Regeneration," vol. 6, 2017, pp. 21-70.
[226] L. A. a. T. R. Fortier, "Stem Cells and Regenerative Therapy," in *Veterinary Surgen Small Animal*, E. Saunders, Ed., ed, 2012, pp. 40-42.
[227] S. Gioventu *et al.*, "A novel method for banking dental pulp stem cells," *Transfus Apher Sci,* vol. 47, no. 2, pp. 199-206, Oct 2012.
[228] N. A, "Ten cate's oral histology-pageburst on vitalsource: development, structure, and function:," *Elsevier Health Sciences,* 2007.
[229] S. Shi and S. Gronthos, "Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp," (in eng), *J Bone Miner Res,* vol. 18, no. 4, pp. 696-704, Apr 2003.
[230] I. Kerkis *et al.*, "Early transplantation of human immature dental pulp stem cells from baby teeth to golden retriever muscular dystrophy (GRMD) dogs: Local or systemic?," *J Transl Med,* vol. 6, p. 35, 2008.
[231] D. Ma *et al.*, "Effect of age and extrinsic microenvironment on the proliferation and osteogenic differentiation of rat dental pulp stem cells in vitro," (in eng), *J Endod,* vol. 35, no. 11, pp. 1546-53, Nov 2009.
[232] S. Tomic *et al.*, "Immunomodulatory properties of mesenchymal stem cells derived from dental pulp and dental follicle are susceptible to activation by toll-like receptor agonists," (in eng), *Stem Cells Dev,* vol. 20, no. 4, pp. 695-708, Apr 2011.
[233] G. Laino *et al.*, "An approachable human adult stem cell source for hard-tissue engineering," *J Cell Physiol,* vol. 206, no. 3, pp. 693-701, Mar 2006.
[234] I. Kerkis and A. I. Caplan, "Stem cells in dental pulp of deciduous teeth," *Tissue Eng Part B Rev,* vol. 18, no. 2, pp. 129-38, Apr 2012.
[235] V. Govindasamy *et al.*, "Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth," *J Endod,* vol. 36, no. 9, pp. 1504-15, Sep 2010.
[236] J. Suchánek *et al.*, "Human dental pulp stem cells--isolation and long term cultivation," *Acta medica (Hradec Králové)/Universitas Carolina, Facultas Medica Hradec Králové,* no. 50, pp. 195-201, 2007.
[237] J. Karbanova, T. Soukup, J. Suchanek, R. Pytlik, D. Corbeil, and J. Mokry, "Characterization of dental pulp stem cells from impacted third molars cultured in low serum-containing medium," *Cells Tissues Organs,* vol. 193, no. 6, pp. 344-65, 2011.
[238] A. H. Huang, Y. K. Chen, L. M. Lin, T. Y. Shieh, and A. W. Chan, "Isolation and characterization of dental pulp stem cells from a supernumerary tooth," *J Oral Pathol Med,* vol. 37, no. 9, pp. 571-4, Oct 2008.
[239] A. H. Huang, Y. K. Chen, A. W. Chan, T. Y. Shieh, and L. M. Lin, "Isolation and characterization of human dental pulp stem/stromal cells from nonextracted crown-fractured teeth requiring root canal therapy," *J Endod,* vol. 35, no. 5, pp. 673-81, May 2009.
[240] B. C. Perry *et al.*, "Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use," *Tissue Eng Part C Methods,* vol. 14, no. 2, pp. 149-56, Jun 2008.
[241] R. J. Waddington, S. J. Youde, C. P. Lee, and A. J. Sloan, "Isolation of distinct progenitor stem cell populations from dental pulp," *Cells Tissues Organs,* vol. 189, no. 1-4, pp. 268-74, 2009.
[242] A. Bakopoulou *et al.*, "Comparative analysis of in vitro osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP)," *Arch Oral Biol,* vol. 56, no. 7, pp. 709-21, Jul 2011.

[243] J. Yu *et al.*, "Differentiation potential of STRO-1+ dental pulp stem cells changes during cell passaging," *BMC Cell Biol,* vol. 11, p. 32, 2010.
[244] S. Shi, P. Robey, and S. Gronthos, "Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis," *Bone,* vol. 29, no. 6, pp. 532-539, 2001.
[245] N. Wada, D. Menicanin, S. Shi, P. M. Bartold, and S. Gronthos, "Immunomodulatory properties of human periodontal ligament stem cells," *J Cell Physiol,* vol. 219, no. 3, pp. 667-76, Jun 2009.
[246] T. Struys, M. Moreels, W. Martens, R. Donders, E. Wolfs, and I. Lambrichts, "Ultrastructural and immunocytochemical analysis of multilineage differentiated human dental pulp- and umbilical cord-derived mesenchymal stem cells," *Cells Tissues Organs,* vol. 193, no. 6, pp. 366-78, 2011.
[247] S. Y. Lee *et al.*, "Effects of cryopreservation of intact teeth on the isolated dental pulp stem cells," *J Endod,* vol. 36, no. 8, pp. 1336-40, Aug 2010.
[248] X. Wei, J. Ling, L. Wu, L. Liu, and Y. Xiao, "Expression of mineralization markers in dental pulp cells," *J Endod,* vol. 33, no. 6, pp. 703-8, Jun 2007.
[249] M. Atari *et al.*, "Isolation of pluripotent stem cells from human third molar dental pulp," (in eng), *Histol Histopathol,* vol. 26, no. 8, pp. 1057-70, Aug 2011.
[250] J. Y. Lee *et al.*, "The effects of platelet-rich plasma derived from human umbilical cord blood on the osteogenic differentiation of human dental stem cells," *In Vitro Cell Dev Biol Anim,* vol. 47, no. 2, pp. 157-64, Feb 2011.
[251] G. Papaccio *et al.*, "Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair," *J Cell Physiol,* vol. 208, no. 2, pp. 319-25, Aug 2006.
[252] A. Arthur, G. Rychkov, S. Shi, S. A. Koblar, and S. Gronthos, "Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues," *Stem Cells,* vol. 26, no. 7, pp. 1787-95, Jul 2008.
[253] W. L. Dissanayaka, X. Zhu, C. Zhang, and L. Jin, "Characterization of dental pulp stem cells isolated from canine premolars," *J Endod,* vol. 37, no. 8, pp. 1074-80, Aug 2011.
[254] X. Struillou, H. Boutigny, A. Soueidan, and P. Layrolle, "Experimental animal models in periodontology: a review," *The open dentistry journal,* vol. 4, p. 37, 2010.
[255] B. E. Grottkau, P. P. Purudappa, and Y. f. Lin, "Multilineage differentiation of dental pulp stem cells from green fluorescent protein transgenic mice," *International journal of oral science,* vol. 2, no. 1, p. 21, 2010.
[256] A. Balic, H. L. Aguila, M. J. Caimano, V. P. Francone, and M. Mina, "Characterization of stem and progenitor cells in the dental pulp of erupted and unerupted murine molars," *Bone,* vol. 46, no. 6, pp. 1639-1651, 2010.
[257] A. Balic and M. Mina, "Characterization of progenitor cells in pulps of murine incisors," *Journal of dental research,* vol. 89, no. 11, pp. 1287-1292, 2010.
[258] S. Kermani, R. M. A. Wahab, I. Z. Z. Abidin, Z. Z. Ariffin, S. Senafi, and S. H. Z. Ariffin, "Differentiation capacity of mouse dental pulp stem cells into osteoblasts and osteoclasts," *Cell Journal (Yakhteh),* vol. 16, no. 1, p. 31, 2014.
[259] M. N. Akmal, Z. I. Zarina, M. Rohaya, S. Sahidan, Z. Zaidah, and Z. S. Hisham, "Isolation and characterization of dental pulp stem cells from murine incisors," *Journal of Biological Sciences,* vol. 14, no. 4, pp. 327-331, 2014.
[260] K. M. Ellis, D. C. O'Carroll, M. D. Lewis, G. Y. Rychkov, and S. A. Koblar, "Neurogenic potential of dental pulp stem cells isolated from murine incisors," *Stem cell research & therapy,* vol. 5, no. 1, p. 30, 2014.
[261] H. Nakamura, L. Saruwatari, H. Aita, K. Takeuchi, and T. Ogawa, "Molecular and biomechanical characterization of mineralized tissue by dental pulp cells on titanium," *Journal of dental research,* vol. 84, no. 6, pp. 515-520, 2005.

[262] M. Patel, A. J. Smith, A. J. Sloan, G. Smith, and P. R. Cooper, "Phenotype and behaviour of dental pulp cells during expansion culture," *Arch Oral Biol,* vol. 54, no. 10, pp. 898-908, Oct 2009.
[263] E. T. Guimarães *et al.*, "Mesenchymal and embryonic characteristics of stem cells obtained from mouse dental pulp," *Archives of oral biology,* vol. 56, no. 11, pp. 1247-1255, 2011.
[264] R. M. El-Backly, A. G. Massoud, A. M. El-Badry, R. A. Sherif, and M. K. Marei, "Regeneration of dentine/pulp-like tissue using a dental pulp stem cell/poly (lactic-co-glycolic) acid scaffold construct in New Zealand white rabbits," *Australian Endodontic Journal,* vol. 34, no. 2, pp. 52-67, 2008.
[265] H.-C. Liu *et al.*, "Reconstruction of alveolar bone defects using bone morphogenetic protein 2 mediated rabbit dental pulp stem cells seeded on nano-hydroxyapatite/collagen/poly (L-lactide)," *Tissue Engineering Part A,* vol. 17, no. 19-20, pp. 2417-2433, 2011.
[266] N. Homayounfar *et al.*, "Isolation, characterization, and differentiation of dental pulp stem cells in ferrets," *Journal of endodontics,* vol. 42, no. 3, pp. 418-424, 2016.
[267] K. Iohara *et al.*, "Regeneration of dental pulp after pulpotomy by transplantation of CD31-/CD146-side population cells from a canine tooth," 2009.
[268] Y. Yamada, K. Ito, S. Nakamura, M. Ueda, and T. Nagasaka, "Promising cell-based therapy for bone regeneration using stem cells from deciduous teeth, dental pulp, and bone marrow," *Cell transplantation,* vol. 20, no. 7, pp. 1003-1013, 2011.
[269] Y. Yamada *et al.*, "A feasibility of useful cell-based therapy by bone regeneration with deciduous tooth stem cells, dental pulp stem cells, or bone-marrow-derived mesenchymal stem cells for clinical study using tissue engineering technology," *Tissue Engineering Part A,* vol. 16, no. 6, pp. 1891-1900, 2010.
[270] K. Iohara, L. Zheng, M. Ito, A. Tomokiyo, K. Matsushita, and M. Nakashima, "Side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis," *Stem cells,* vol. 24, no. 11, pp. 2493-2503, 2006.
[271] Y. Zheng *et al.*, "Stem cells from deciduous tooth repair mandibular defect in swine," *Journal of dental research,* vol. 88, no. 3, pp. 249-254, 2009.
[272] P. H. Cheng, B. Snyder, D. Fillos, C. C. Ibegbu, A. H. Huang, and A. W. Chan, "Postnatal stem/progenitor cells derived from the dental pulp of adult chimpanzee," *BMC Cell Biol,* vol. 9, p. 20, 2008.
[273] S. Gronthos, K. Mrozik, S. Shi, and P. M. Bartold, "Ovine periodontal ligament stem cells: isolation, characterization, and differentiation potential," *Calcif Tissue Int,* vol. 79, no. 5, pp. 310-7, Nov 2006.
[274] K. M. Mrozik *et al.*, "Proteomic characterization of mesenchymal stem cell-like populations derived from ovine periodontal ligament, dental pulp, and bone marrow: analysis of differentially expressed proteins," *Stem cells and development,* vol. 19, no. 10, pp. 1485-1499, 2010.
[275] J. B. Sakdee, R. R. White, T. C. Pagonis, and P. V. Hauschka, "Hypoxia-amplified proliferation of human dental pulp cells," *J Endod,* vol. 35, no. 6, pp. 818-23, Jun 2009.
[276] "<d'Aquino et al., 2009.pdf>."
[277] L. Spath *et al.*, "Explant-derived human dental pulp stem cells enhance differentiation and proliferation potentials," *Journal of Cellular and Molecular Medicine* vol. 14, no. 6B, pp. 1635-44, Jun 2010.
[278] H. Ikeda *et al.*, "Engineering bone formation from human dental pulp- and periodontal ligament-derived cells," *Ann Biomed Eng,* vol. 39, no. 1, pp. 26-34, Jan 2011.
[279] L. Pierdomenico *et al.*, "Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp," *Transplantation,* vol. 80, no. 6, pp. 836-842, 2005.
[280] B. Alliot-Licht *et al.*, "Dexamethasone stimulates differentiation of odontoblast-like cells in human dental pulp cultures," *Cell Tissue Res,* vol. 321, no. 3, pp. 391-400, Sep 2005.

[281] N. F. Lizier *et al.*, "Scaling-up of dental pulp stem cells isolated from multiple niches," *PLoS One,* vol. 7, no. 6, p. e39885, 2012.
[282] K. Iida, T. Takeda-Kawaguchi, Y. Tezuka, T. Kunisada, T. Shibata, and K. Tezuka, "Hypoxia enhances colony formation and proliferation but inhibits differentiation of human dental pulp cells," *Arch Oral Biol,* vol. 55, no. 9, pp. 648-54, Sep 2010.
[283] T. M. Hirata *et al.*, "Expression of multiple stem cell markers in dental pulp cells cultured in serum-free media," *J Endod,* vol. 36, no. 7, pp. 1139-44, Jul 2010.
[284] M. Atari *et al.*, "Isolation of pluripotent stem cells from human third molar dental pulp," *Histology and histopathology,* vol. 26, no. 7, p. 1057, 2011.
[285] B. Chan, R. W. Wong, and B. Rabie, "In vivo production of mineralised tissue pieces for clinical use: a qualitative pilot study using human dental pulp cell," *Int J Oral Maxillofac Surg,* vol. 40, no. 6, pp. 612-20, Jun 2011.
[286] W. Zhang *et al.*, "In vivo evaluation of human dental pulp stem cells differentiated towards multiple lineages," *J Tissue Eng Regen Med,* vol. 2, no. 2-3, pp. 117-25, Mar-Apr 2008.
[287] W. Zhang, X. F. Walboomers, S. Shi, M. Fan, and J. A. Jansen, "Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation," *Tissue engineering,* vol. 12, no. 10, pp. 2813-2823, 2006.
[288] C. Zhang, J. Chang, W. Sonoyama, S. Shi, and C.-Y. Wang, "Inhibition of human dental pulp stem cell differentiation by Notch signaling," *Journal of dental research,* vol. 87, no. 3, pp. 250-255, 2008.
[289] E. J. Woods, B. C. Perry, J. J. Hockema, L. Larson, D. Zhou, and W. S. Goebel, "Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use," (in eng), *Cryobiology,* vol. 59, no. 2, pp. 150-7, Oct 2009.
[290] F. Ferro, R. Spelat, A. P. Beltrami, D. Cesselli, and F. Curcio, "Isolation and characterization of human dental pulp derived stem cells by using media containing low human serum percentage as clinical grade substitutes for bovine serum," *PLoS One,* vol. 7, no. 11, p. e48945, 2012.
[291] J. Karbanová, T. Soukup, J. Suchánek, R. Pytlík, D. Corbeil, and J. Mokrý, "Characterization of dental pulp stem cells from impacted third molars cultured in low serum-containing medium," *Cells Tissues Organs,* vol. 193, no. 6, p. 344, 2011.
[292] A. de Mendonca Costa *et al.*, "Reconstruction of large cranial defects in nonimmunosuppressed experimental design with human dental pulp stem cells," (in eng), *J Craniofac Surg,* vol. 19, no. 1, pp. 204-10, Jan 2008.
[293] C. Mangano *et al.*, "Human dental pulp stem cells hook into biocoral scaffold forming an engineered biocomplex," *PloS one,* vol. 6, no. 4, p. e18721, 2011.
[294] F. Paino *et al.*, "Histone deacetylase inhibition with valproic acid downregulates osteocalcin gene expression in human dental pulp stem cells and osteoblasts: evidence for HDAC2 involvement," (in eng), *Stem Cells,* vol. 32, no. 1, pp. 279-89, Jan 2014.
[295] F. F. Demarco *et al.*, "Effects of morphogen and scaffold porogen on the differentiation of dental pulp stem cells," *J Endod,* vol. 36, no. 11, pp. 1805-11, Nov 2010.
[296] R. S. Prescott *et al.*, "In vivo generation of dental pulp-like tissue by using dental pulp stem cells, a collagen scaffold, and dentin matrix protein 1 after subcutaneous transplantation in mice," *J Endod,* vol. 34, no. 4, pp. 421-6, Apr 2008.
[297] C. H. Liu, C. J. Hung, T. H. Huang, C. C. Lin, C. T. Kao, and M. Y. Shie, "Odontogenic differentiation of human dental pulp cells by calcium silicate materials stimulating via FGFR/ERK signaling pathway," (in eng), *Mater Sci Eng C Mater Biol Appl,* vol. 43, pp. 359-66, Oct 2014.
[298] J. Liu, T. Jin, S. Chang, H. H. Ritchie, A. J. Smith, and B. H. Clarkson, "Matrix and TGF-beta-related gene expression during human dental pulp stem cell (DPSC) mineralization," (in eng), *In Vitro Cell Dev Biol Anim,* vol. 43, no. 3-4, pp. 120-8, Mar-Apr 2007.
[299] J. R. Perez, D. Kouroupis, D. J. Li, T. M. Best, L. Kaplan, and D. Correa, "Tissue Engineering and Cell-Based Therapies for Fractures and Bone Defects," *Frontiers in bioengineering and biotechnology,* vol. 6, pp. 105-105, 2018.

[300] W. L. Murphy, M. C. Peters, D. H. Kohn, and D. J. Mooney, "Sustained release of vascular endothelial growth factor from mineralized poly(lactide-co-glycolide) scaffolds for tissue engineering," (in eng), *Biomaterials,* vol. 21, no. 24, pp. 2521-7, Dec 2000.
[301] A. Graziano *et al.*, "Scaffold's surface geometry significantly affects human stem cell bone tissue engineering," (in eng), *J Cell Physiol,* vol. 214, no. 1, pp. 166-72, Jan 2008.
[302] X. Yang, F. Yang, X. F. Walboomers, Z. Bian, M. Fan, and J. A. Jansen, "The performance of dental pulp stem cells on nanofibrous PCL/gelatin/nHA scaffolds," (in eng), *J Biomed Mater Res A,* vol. 93, no. 1, pp. 247-57, Apr 2010.
[303] W. Zhang, X. F. Walboomers, T. H. van Kuppevelt, W. F. Daamen, Z. Bian, and J. A. Jansen, "The performance of human dental pulp stem cells on different three-dimensional scaffold materials," (in eng), *Biomaterials,* vol. 27, no. 33, pp. 5658-68, Nov 2006.
[304] X. Wang *et al.*, "Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells," *Arch Oral Biol,* vol. 57, no. 9, pp. 1231-40, Sep 2012.
[305] T. Yamaza *et al.*, "Immunomodulatory properties of stem cells from human exfoliated deciduous teeth," *Stem cell research & therapy,* vol. 1, no. 1, pp. 5-5, 2010.
[306] B. M. Seo *et al.*, "SHED repair critical-size calvarial defects in mice," (in eng), *Oral Dis,* vol. 14, no. 5, pp. 428-34, Jul 2008.
[307] X. Yang, P. M. van der Kraan, Z. Bian, M. Fan, X. F. Walboomers, and J. A. Jansen, "Mineralized tissue formation by BMP2-transfected pulp stem cells," (in eng), *J Dent Res,* vol. 88, no. 11, pp. 1020-5, Nov 2009.
[308] S. Abe, S. Yamaguchi, and T. Amagasa, "Multilineage Cells from Apical Pulp of Human Tooth with Immature Apex," *Oral Science International,* vol. 4, no. 1, pp. 45-58, 2007/05/01/ 2007.
[309] S. R. Motamedian, F. S. Tabatabaei, F. Akhlaghi, M. Torshabi, P. Gholamin, and A. Khojasteh, "Response of Dental Pulp Stem Cells to Synthetic, Allograft, and Xenograft Bone Scaffolds," (in eng), *Int J Periodontics Restorative Dent,* Aug 24 2016.
[310] Y. Okamoto *et al.*, "Simvastatin induces the odontogenic differentiation of human dental pulp stem cells in vitro and in vivo," (in eng), *J Endod,* vol. 35, no. 3, pp. 367-72, Mar 2009.
[311] S. Gronthos, "The therapeutic potential of dental pulp cells: more than pulp fiction?," (in eng), *Cytotherapy,* vol. 13, no. 10, pp. 1162-3, Nov 2011.
[312] C. Morsczeck *et al.*, "In vitro differentiation of human dental follicle cells with dexamethasone and insulin," (in eng), *Cell Biol Int,* vol. 29, no. 7, pp. 567-75, Jul 2005.
[313] E. W. Bachtiar, L. R. Amir, P. Suhardi, and B. Abas, "Scaffold degradation during bone tissue reconstruction in Macaca nemestrina mandible," *Interventional medicine & applied science,* vol. 8, no. 2, pp. 77-81, 2016.
[314] J. D. Hartgerink, "Covalent capture: a natural complement to self-assembly," *Curr Opin Chem Biol,* vol. 8, no. 6, pp. 604-9, Dec 2004.
[315] H. Hosseinkhani, M. Hosseinkhani, F. Tian, H. Kobayashi, and Y. Tabata, "Osteogenic differentiation of mesenchymal stem cells in self-assembled peptide-amphiphile nanofibers," *Biomaterials,* vol. 27, no. 22, pp. 4079-86, Aug 2006.
[316] M. O. Guler, L. Hsu, S. Soukasene, D. A. Harrington, J. F. Hulvat, and S. I. Stupp, "Presentation of RGDS epitopes on self-assembled nanofibers of branched peptide amphiphiles," *Biomacromolecules,* vol. 7, no. 6, pp. 1855-63, Jun 2006.
[317] K. M. Galler, J. D. Hartgerink, A. C. Cavender, G. Schmalz, and R. N. D'Souza, "A customized self-assembling peptide hydrogel for dental pulp tissue engineering," *Tissue Eng Part A,* vol. 18, no. 1-2, pp. 176-84, Jan 2012.
[318] K. M. Galler, R. N. D'Souza, J. D. Hartgerink, and G. Schmalz, "Scaffolds for dental pulp tissue engineering," *Adv Dent Res,* vol. 23, no. 3, pp. 333-9, Jul 2011.
[319] K. M. Galler *et al.*, "Self-assembling peptide amphiphile nanofibers as a scaffold for dental stem cells," (in eng), *Tissue Eng Part A,* vol. 14, no. 12, pp. 2051-8, Dec 2008.

[320] M. Riccio *et al.*, "Fibroin scaffold repairs critical-size bone defects in vivo supported by human amniotic fluid and dental pulp stem cells," *Tissue Eng Part A,* vol. 18, no. 9-10, pp. 1006-13, May 2012.
[321] S. Z. Khan, E. Kokubu, K. Matsuzaka, and T. Inoue, "Behaviour of rat-cultured dental pulp cells in three-dimensional collagen type-1 gel in vitro and in vivo," (in eng), *Aust Endod J,* vol. 39, no. 3, pp. 137-45, Dec 2013.
[322] R. d'Aquino *et al.*, "Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes," (in eng), *Eur Cell Mater,* vol. 18, pp. 75-83, Nov 12 2009.
[323] R. d'Aquino *et al.*, "Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation," *Cell Death Differ,* vol. 14, no. 6, pp. 1162-71, Jun 2007.
[324] C. A. Gregory, W. G. Gunn, A. Peister, and D. J. Prockop, "An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction," *Analytical biochemistry,* vol. 329, no. 1, pp. 77-84, 2004.
[325] A. I. Pearce, R. G. Richards, S. Milz, E. Schneider, and S. G. Pearce, "Animal models for implant biomaterial research in bone: A review," *European Cells and Materials,* vol. 13, pp. 1-10, 2007.
[326] V. D, C. M, O.-V. I, and V. J, "Derivation of Multipotent Mesenchymal Stromal Cells from Ovine Bone Marrow.," *Curr Protoc Stem Cell Biol* vol. 44, 2018.
[327] L. Martini, M. Fini, G. Giavaresi, and R. Giardino, "Sheep model in orthopedic research: a literature review," *Comparative Medicine,* vol. 51, no. 4, pp. 292-299, 2001.
[328] J. Aerssens, S. Boonen, J. Joly, and J. Dequeker, "Variations in trabecular bone composition with anatomical site and age: Potential implications for bone quality assessment," *Journal of Endocrinology,* vol. 155, no. 3, pp. 411-21, 1998.
[329] TISSEEL, "FOLHETO INFORMATIVO: INFORMAÇÃO PARA O UTILIZADOR - TISSEEL," INFARMED, Ed., ed, 2013.
[330] A. M. Beck and N. M. Meyers, "Health enhancement and companion animal ownership," *Annual review of public health,* vol. 17, no. 1, pp. 247-257, 1996.
[331] J. M.R., "Introduction: Children and the Dogs in Their Lives.," in *Children, Dogs and Education.* , vol. 1, C. Springer, Ed. In: Renck Jalongo M. (eds) ed., 2018.
[332] A. Mazzatenta, A. Carluccio, D. Robbe, C. D. Giulio, and A. Cellerino, "The companion dog as a unique translational model for aging.," *Seminars in Cell & Developmental Biology,* vol. 70, pp. 141-153,, 2017.
[333] J. Franch, C. Diaz-Bertrana, P. Lafuente, P. Fontecha, and I. Durall, "Beta-tricalcium phosphate as a synthetic cancellous bone graft in veterinary orthopaedics: a retrospective study of 13 clinical cases," (in eng), *Vet Comp Orthop Traumatol,* vol. 19, no. 4, pp. 196-204, 2006.
[334] M. S. Si, J. J. Mo, L. F. Zhuang, Y. X. Gu, S. C. Qiao, and H. C. Lai, "Osteotome sinus floor elevation with and without grafting: an animal study in Labrador dogs," (in eng), *Clin Oral Implants Res,* vol. 26, no. 2, pp. 197-203, Feb 2015.
[335] L. Atayde, P. Cortez, A. Afonso, M. Santos, A. Maurício, and J. Santos, "Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike®) on osteoregeneration," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 103, no. 2, pp. 292-304, 2015.
[336] J. V. Lobato, N. S. Hussain, and C. M. Botelho, "Assessment of the Potential of Bonelike® Graft for Bone Regeneration by using an Animal Model," *Key Engineering Materials,* vol. 284-286, pp. 877-880, 2005.
[337] R. C. Sousa *et al.*, "A clinical report of bone regeneration in maxillofacial surgery using bonelike synthetic bone graft," (in eng), *J Biomater Appl,* vol. 22, no. 4, pp. 373-85, Jan 2008.
[338] G. T. Huang, S. Gronthos, and S. Shi, "Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine," (in eng), *J Dent Res,* vol. 88, no. 9, pp. 792-806, Sep 2009.

[339] J. Oliveira, T. Kawai, M. Lopes, C. Ohtsuki, J. D. Santos, and A. Afonso, *In Vivo Behaviour of Bonelike ® /PLGA Hybrid: Histological Analysis and Peripheral Quantitative Computed Tomography (pQ-CT) Evaluation*. 2004, pp. 565-568.

[340] B. A and C. U, "Maxillary alveolar ridge reconstruction with nonvascularized autogenous block bone: clinical results.," *J Oral Maxillofac Surg. ,* vol. 65, no. 10, pp. 2039-46, 2007

[341] Keating J and M. MM., "Substitutes for autologous bone graft in orthopaedic trauma.," *J Bone Joint Surg Br* vol. 83, no. 5, p. 777, 2001.

[342] Nkenke E, Radespiel-Tröger M, Wiltfang J, Schultze-Mosgau S, Winkler G, and N. FW, "Morbidity of harvesting of retromolar bone grafts: a prospective study.," *Clin Oral Implants Res. ,* vol. 13, no. 5, pp. 514-21, 2002.

[343] Sawada K *et al.*, "Comparison of three block bone substitutes for bone regeneration: long-term observation in the beagle dog.," *Odontology -Official Journal of the Society of the Nippon Dental University,* 2018. Springer Japan

[344] M. A. Lopes *et al.*, "Push-out testing and histological evaluation of glass reinforced hydroxyapatite composites implanted in the tibia of rabbits," (in eng), *J Biomed Mater Res,* vol. 54, no. 4, pp. 463-9, Mar 15 2001.

[345] A. Graziano *et al.*, "Scaffold's surface geometry significantly affects human stem cell bone tissue engineering," *Journal of cellular physiology,* vol. 214, no. 1, pp. 166-172, 2008.

[346] A. Caseiro *et al.*, *Human Umbilical Cord Blood Plasma as an Alternative to Animal Sera for Mesenchymal Stromal Cells In vitro Expansion – a Multicomponent Metabolomic Analysis (IN PRESS Manuscript ID:PONE-D-18-02368R2)*. 2018.

ATTACHMENTS

ICBAS
UPORTO

CIRURGIA EXPERIMENTAL

Nr Brinco DGV PT _____

CIRURGIA - -2017 /ABATE - - (TEMPO Mês)

Ovelha (raça:_____ Idade Aprox.:_____)

Anestesia Total:__ml Xilazina/IM + {____ml + ___ml + __ml Tiletamina+Zolazepam/IV} + Epidural ___ml Lidocaina

Medicação: (___ml Penicilina+Estreptomicina + ___ml Meloxicaml no pos-op) x____dias.

Membro Posterior DIREITO			
Orifício nr. 1 (proximal)	----------	----------	----------
Orifício nr. 2	Bonelike	Tissel	DPSCs (__µl x10^5)
Orifício nr. 3	Bonelike	Tisseel	DPSCs (__µl x10^5)
Orifício nr. 4	Bonelike	Tisseel	DPSCs (__µl x10^5)
Orifício nr. 5 (distal)	Bonelike	Tisseel	DPSCs (__µl x10^5)
Membro Posterior ESQUERDO			
Orifício nr. 1 (proximal)	----------	----------	----------
Orifício nr. 2	Bonelike	Tissel	DPSCs (__µl x10^5)
Orifício nr. 3	Bonelike	Tisseel	DPSCs (__µl x10^5)
Orifício nr. 4	Bonelike	Tisseel	DPSCs (__µl x10^5)
Orifício nr. 5 (distal)	Bonelike	Tisseel	DPSCs (__µl x10^5)

OBSERVAÇÕES:

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U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

FICHA DIÁRIA DE INTERNAMENTO

Centro Clínico e de Investigação Veterinária de Vairão

PROJETOS DE DOCÊNCIA E INVESTIGAÇÃO

Espécie: _____ **Raça:** _____ **Idade:**_____ **Local de internamento do animal:**_____

DATA ACTUAL: / / DATA ENTRADA: / / CIRURGIA: VET. RESP.:	LOCAL CATETER:_____ DATA COLOCAÇÃO:__/__/__ CLÍNICO:_____	ALIMENTAÇAO: _____ PESO ACTUAL: _____ PESO ENTRADA: _____	BRINCO NÚMERO: _____

SUMARIO CLINICO:

Procedimentos:	HORA	1	2	3	4	5	6	7	8	9	10	11	12
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EXAME CLINICO:

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Mucosas: TRC:
Pulso:
Temp. Retal:
Palpação abdominal:
Hidratação:
FR: FC:
Auscultação toracica:
Palpação ganglionar:
Estado da ferida cirúrgica:

OBSERVAÇÕES/ PLANO TERAPÊUTICO DO DIA/ COMUNICAÇÕES:

Em colaboração com

Hospital Veterinário Universitário de Coimbra
Escola Universitária Vasco da Gama

CONSENTIMENTO INFORMADO

LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO EM
PROJETOS DE DOCÊNCIA E/OU INVESTIGAÇÃO

Por favor, leia com atenção a seguinte informação. Se achar que algo está incorreto ou que não está claro, não hesite em solicitar mais informações. Se concorda com a proposta que lhe foi feita, queira assinar este documento.

Enquadramento: Estudo no âmbito da docência/investigação clinica a realizar no Hospital Veterinário Universitário de Coimbra, em colaboração com o Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Título do estudo: "Desenvolvimento de Biomateriais e Terapias Celulares para Regeneração Óssea"

Explicação do estudo: O substituto ósseo BL® foi testado em inúmeras situações clínicas e tem provado a sua excelente biocompatibilidade e bioatividade, conforme comprovam os ensaios clínicos realizados em animais e humanos. Este substituto ósseo sintético, produzido com base numa mistura de hidroxiapatite e fosfato tricálcico, pode ser utilizado em situações clínicas de regeneração óssea, já tendo sido testado nomeadamente no preenchimento de defeitos ósseos após a extração de quistos, em implantologia, após a remoção cirúrgica de dentes inclusos, no preenchimento de bolsas ósseas, assim como em bifurcações ou trifurcações de dentes, na elevação óssea do seio maxilar e em aplicações ortopédicas. Contudo, em inúmeras situações clínicas e, em particular, nas doenças ósseas mais graves, como acontece com pacientes de idade avançada e/ou que sofram de osteoporose, torna-se absolutamente necessário a adoção de novas metodologias de preparação e

aplicação dos substitutos ósseos. Neste enquadramento, verifica-se a necessidade de desenvolver e otimizar estes substitutos ósseos, com a introdução de veículos à base de estabilizadores poliméricos adequados à regeneração do tecido ósseo, facilitando a aplicação simultânea de moléculas terapêuticas ou de microelementos favoráveis a essa regeneração. Desta forma, permitir-se-á a libertação lenta destas moléculas, favorecendo a manutenção de uma concentração fisiológica no local da sua aplicação. Também o uso deste biomaterial combinado com o uso de células estaminais permite acelerar o processo de cicatrização óssea sendo que o seu uso conjunto nunca foi estudado e portanto pretende por nós ser investigado em ensaios clínicos em canídeos.

Material a estudo: Testar *in vivo* (casos clínicos de canídeos) o substituto ósseo patenteado Bonelike® (J.D Santos, G.W Hastings, J.C.Knowles, European Patent WO 0068164) na forma de esferas, de 250-500 µm de diâmetro, associados a um veículo bioabsorvível (estabilizador polimérico). Este gel servirá para melhorar a aplicação e a compactação das esferas nos defeitos ósseos, além de funcionar como veículo para a libertação lenta das moléculas terapêuticas a serem testadas como é o caso das células estaminais.

Condições e financiamento: Neste estudo não existe pagamento de contrapartidas. O tratamento de dados ou análises laboratoriais de amostras biológicas deste estudo não envolverão qualquer custo adicional para o proprietário do animal e todas despesas provenientes do estudo serão custeadas pelos docentes/investigadores responsáveis. A participação neste estudo é voluntária e não existe qualquer prejuízo caso não queira participar.

Este estudo foi submetido à ORBEA do ICBAS-UP e mereceu um parecer favorável.

Confidencialidade e anonimato: Todos os dados recolhidos serão utilizados exclusivamente neste estudo/ensaio clinico e será garantida confidencialidade sobre a origem das amostras, apenas sendo indicadas informações relativas ao paciente que sejam fundamentais para a posterior analise de dados (*eg* idade do animal, raça, parâmetros vitais, informação da anamnese, etc).

Agradecimentos e identificação do/a investigador/a e da pessoa que pede o consentimento, se for diferente:

Clinico(a)/Investigador(a) responsável do projeto:

Professora Doutora Ana Colette Maurício

Clinico(a)/Investigador(a):

Mestre José Miguel Campos

Expressamos o nosso agradecimento pela sua participação e contribuição neste estudo.

Assinatura(s):

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Declaro ter lido e compreendido este documento, bem como as informações verbais que me foram fornecidas pela/s pessoas/s que acima assina/m e que considero suficientes. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Desta forma, aceito participar neste estudo e permito a utilização dos dados que de forma voluntária forneço, confiando em que apenas serão utilizados para esta investigação e nas garantias de confidencialidade e anonimato que me são dadas pelo/a investigador/a.

Nome: _____

Número de cartão de cidadão: _____

Assinatura: Data: __ /__ /_____

ESTE DOCUMENTO, COMPOSTO DE 2 PÁGINAS, É FEITO EM DUPLICADO:
UMA VIA PARA O/A INVESTIGADOR/A, OUTRA PARA A PESSOA QUE CONSENTE
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7th–10th of April 2016 – **Poster Presentation** at 59th Congress of British Small Veterinary Association (BSAVA), Birmingham, England, United Kingdom.

HVUC Hospital Veterinário Universitário de Coimbra

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U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

BSAVA Congress, 7-10th April 2016, Birmingham, UK

Use of a glass-reinforced hydroxyapatite synthetic bone in small animal clinical cases

José Miguel Campos, Pedro Pinto, Rita Caseiro, Tiago Pereira, Luís Atayde, Jorge Ribeiro, Miguel França, Carla Mendonça, José Santos, Ana Colette Maurício

ICBAS/ICETA - Center for Animal Science - University of Porto, Portugal

Aims & Objectives:

This pre-clinical trial intends to contribute to the development of new strategies, in the context of bone regeneration surgery, targeting a rapid restoration of the skeletal integrity and reduction of the associated morbidity where biomaterials such as ceramics play an important role.



Figure 1 & 2 - Ceramic biomaterial used: Bonelike® patented bone substitute (JD Santos, GW Hastings, JCKnowles, European Patent WO 0068164)

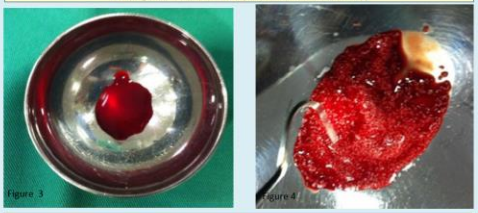


Figure 3 & 4- Autologous blood mixed with Bonelike®

Introduction:

Autologous bone graft remains the gold standard grafting substrate available in bone fusions, for small gaps and in critical defects. Significant morbidity however is associated with the harvesting of autologous bone graft and alternative bone graft substitutes have been developed. In the present pre-clinical study, a synthetic bone graft called Bonelike® (BL®) was tested. BL® is a glass-reinforced hydroxyapatite composite with osteoinductive and osteoconductive proprieties consisting in the incorporation of P_2O_5-CaO glass-based system within a HA matrix of spherical pellets with 250-500µm of diameter (Figure1&2). These proprieties of BL® have been confirmed in experimental models of bone regeneration in sheep and recently its application in orthopedic and stomatological pathologies is being tested in small animal (SA) clinics.

Material & Methods:

In all the SA clinical cases included, BL® was used to enhance bone healing, fulfil critical defects and complement the recommended surgical techniques. BL® is prepared using autologous blood mixed with the spherical pellets (Figure 3&4). In **case #1** the dog mandibular fracture was reduced with external fixation and the fracture consolidation was confirmed 5 weeks after, through radiographic examination (Figure 5). In **case #2** the mandibular fracture was reduced with a plate and BL® fills the bone defect left by the fracture and tooth extraction in a dog (Figure 6). After 8 weeks the consolidation was confirmed. In **case #3** and **#4** BL® was added to the tibial non-union (Figure 7) and to the radio-ulnar non-union (Figure 8) respectively, in a third surgical procedure after both an initial external fixation and subsequent plate fixation failures. Both **case #5** and **#6** were accomplished by filling the alveolar defect created after canine maxillary dental removal, before closing with a mucosal flap in order to prevent oronasal fistulae (Figure 9). Radiologic evaluation and clinical examination were performed in the follow-up periods in order to assess the efficacy of the surgical treatments.

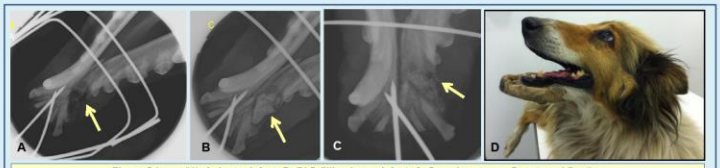


Figure 5 (case #1): A- bone defect; B- BL® filling bone defect; C- 5 weeks pos-op; D- external fixation

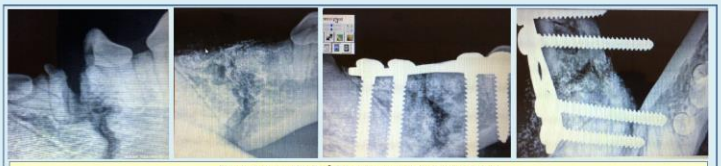


Figure 6 (case #2): BL® filling fracture and alveolar defect



Figure 7 (cases #3): BL® surgery correction sequence after an implant failure

Results & Discussion:

The autograft is associated with prolonged anesthetic times, limited availability, donor site morbidity (pain, intra-operative blood loss and stress fracture), risk of local infection and predisposition to failure. Other problem is the limited amount of bone graft that can be collected from the patient, and the viability of the cells after harvesting, that limiting its application to critical defects. Synthetic bone substitutes can be used to fill critical voided spaces, can be stored easily and some have osteoinduction properties. Biomaterials also have the ability to adapt to the defect shape and to provide a full osteoconductive function. The granular forms (250-500 µm), like spherical, facilitate the flow of biomaterial being less prone to clumping in the syringe (when compared with polygonal) are suitable for voiding non-critical sized bone defects surgery. Radiologic evaluation and clinical examination were performed in the follow-up periods in order to assess the efficacy of the surgical treatments. In neither case local or systemic reactions were detected. Furthermore, the osteoconductive and osteoinductive properties of BL® have been confirmed in all cases.



Figure 8 (cases #4): BL® filling critical bone defect after a non-union and implant failures



Figure 9 (case #5 & #6): filling of non critical alveolar bone defect and mucosal flap

Conclusions:

Bonelike® is ceramic biomaterial, a glass-reinforced hydroxyapatite composite with osteoinductive and osteoconductive proprieties suitable for using as a synthetic bone grafts in the treatment of bone defects and has proven its excellent biocompatibility and bioactivity. Accordingly, there is a need to develop and optimize bone substitutes, based vehicles suitable for regeneration of bone tissue polymeric stabilizers, facilitating the simultaneous application of therapeutic molecules or microelements favor such regeneration.

Bibliography: Atayde, L.M., et al., Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike®) on osteoregeneration. J Biomed Mater Res B Appl Biomater,2015. 103(2): p. 292-304.

Corresponding author: José Miguel Campos DVM MRCVS PhD student (University of Porto). Email: miguelestevesdecampos@hotmail.com

20th–22nd October 2016 – **Poster Presentation** at 51st Congreso Nacional AVEPA (Asociación de Veterinarios Españoles Especialistas en Pequeños Animales) and X Southern European Veterinarian Conference, Granada, Spain.

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HEALTH & SCIENCE SCHOOL

AVEPA-SEVC Congress, 20-22nd October 2016, Granada, Spain

USE OF A BIOGLASS-REINFORCED HYDROXYAPATITE SYNTHETIC BONE SUBSTITUTE (BONELIKE®) FOR OSTEOREGENERATION OF BONE DEFECTS IN SMALL ANIMAL CLINICAL CASES

José Miguel Campos, Pedro Olivério Pinto, Rita Caseiro, Tiago Pereira, Luís Atayde, Carla Mendonça, José Santos, Ana Colette Maurício

ICBAS - Center for Animal Science - University of Porto, Portugal

Aims & Objectives

This clinical cases intends to contribute to the development of new strategies, in the context of bone regeneration surgery, targeting a rapid restoration of the skeletal integrity and reduction of the associated morbidity where biomaterials such as ceramics play an important role.

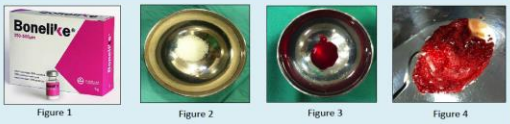


Figure 1 Figure 2 Figure 3 Figure 4

Figure 1 & 2 - Ceramic biomaterial used: Bonelike® patented bone substitute (JD Santos, GW Hastings, JCKnowles, European Patent WO 0068164)
Figure 3 & 4- Autologous blood mixed with Bonelike®

Introduction

Autologous bone graft remains the gold standard grafting substrate available in bone fusions, for small gaps and in critical defects. However the second surgery for harvesting cancellous bone are associated with donor-site morbidity and the amount of bone graft is limited. Bonelike® (BL®) is an alternative synthetic bone graft, a glass-reinforced hydroxyapatite (HA) composite with osteoinductive and osteoconductive proprieties consisting in the incorporation of P_2O_5-CaO glass-based system within a HA matrix of spherical pellets with 250-500µm of diameter (Figure1&2). These proprieties of BL® have been confirmed in experimental models of bone regeneration in sheep and recently its application in orthopedic and stomatological pathologies is being tested in small animal (SA) clinics.

Material & Methods

In all clinical cases included, BL® was used to enhance bone healing, fulfilling critical defects and complement the recommended surgical techniques. BL® is prepared using autologous blood mixed with the spherical pellets (Figure 3 & 4). In **case #1** the mandibular fracture was reduced with a plate and BL® fills the bone defect left by the fracture and tooth extraction in a dog (Figure 5). After 8 weeks the consolidation was confirmed. In **case #2** and **#3** BL® was mixed with autologous cancellous bone graft and platelet rich plasma to treat tibial (Figure 6) and radius-ulnar non-unions (Figure 7), in a revision surgical procedure after an initial external fixation failure. Radiologic evaluation and clinical examination were performed in the follow-up periods in order to assess the efficacy of the surgical treatments.

Results & Discussion

The autograft is associated with prolonged anesthetic times, limited availability, donor site morbidity (pain, intra-operative blood loss and stress fracture), risk of local infection and predisposition to failure. Other problem is the limited amount of bone graft that can be collected from the patient, and the viability of the cells after harvesting, that limiting its application to critical defects. So synthetic bone substitutes can be used to fill critical voided spaces, can be stored easily and some have osteoinduction properties. Biomaterials also have the ability to adapt to the defect shape and to provide a full osteoconductive function. The BL® granular forms (250-500 µm), like spherical, facilitate the flow of biomaterial being less prone to clumping in the syringe are suitable for voiding critical and non-critical sized bone defects surgery.

Clinical results

In case #1 patient start eating within 24h after surgery without any complication associated. In cases #2 and #3 the clinical outcome was considered excellent in both cases with weight-bearing activity starting within in the first 15 days after surgery. Lameness grades improved in the first month and the implanted biomaterials did not cause any apparent signs of irritation, infection or discomfort, and cutaneous wound healing was uneventful.

Radiographic results

Radiologic evaluation and clinical examination were performed in the follow-up periods in order to assess the efficacy of the surgical treatments. In neither case local or systemic reactions were detected. Furthermore, the osteoconductive and osteoinductive properties of BL® have been confirmed in all cases, on a smaller expected time.

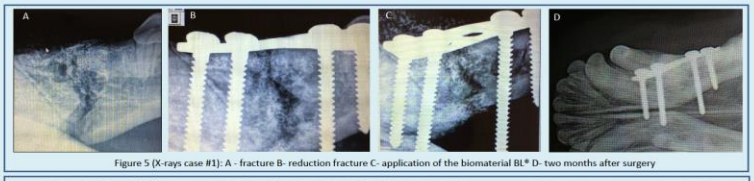


Figure 5 (X-rays case #1): A - fracture B- reduction fracture C- application of the biomaterial BL® D- two months after surgery



Figure 6 (X-rays case #2): A/B/C -tibial mal union after implants failures D/E - BL® application after surgery correction F/G - four months after surgery

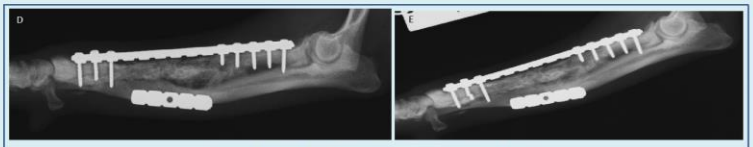


Figure 7 (X-rays case #3): D - four months control E - five months control



Figure 7 (X-rays case #3): F - eight months control G - eleven months control



Figure 7 (X-rays case #3): A – Preoperative (mal union and implant failures)

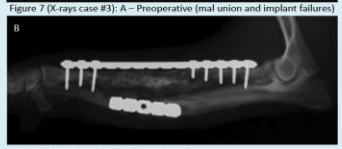




Figure 7 (X-rays case #3): B /C – Postoperative

Dog, male, Sptitz, 5Kg, 1yo and 3 months.

It was used Bonelike (BL®) synthetic bone graft mixed with autologous cancellous bone graft and platelet rich plasma, in right frontlimb (radius/ulna) during corrective ulnar osteotomy, to correct the bone defect of 4.5cm after mal union and implant failures. Imaging reveals denser bone defect area, and a less clear granular aspect of the biomaterial where the empty spaces were filled by material with bone-compatible gray radiographic density.

Conclusions

Bonelike® is ceramic biomaterial, a glass-reinforced hydroxyapatite composite with osteoinductive and osteoconductive proprieties suitable for using as a synthetic bone grafts in the treatment of bone defects and has proven its excellent biocompatibility and bioactivity. All cases herein described resulted in bone healing without any signs of irritation, inflammation or infection, and uneventful wound healing. This biomaterial has proven to closely mimic the characteristics of natural bone, supporting bioactive molecules diffusion and therefore promoting cell proliferation and new bone formation. Accordingly, there is a need to develop and optimize bone substitutes, based vehicles suitable for regeneration of bone tissue polymeric stabilizers, facilitating the simultaneous application of therapeutic molecules or microelements favor such regeneration. Further studies to properly evaluate the molecular mechanisms by which BL® influences the treatment of bone fusion, will be needed and are currently in progress in our group.

Bibliography: Atayde, L.M., et al., Morphology effect of bioglass-reinforced hydroxyapatite (Bonelike®) on osteoregeneration. J Biomed Mater Res B Appl Biomater,2015. 103(2): p. 292-304.

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12th–14th October 2017 – **Poster Presentation** at 10th International Meeting of the Portuguese Society for Stem Cells and Cell Therapies, Covilhã, Portugal.

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Reunião de Inverno da Academia Portuguesa de Medicina Oral, ESTeSL, IPL, Lisboa

BONELIKE® AND CELLULAR THERAPIES FOR REGENERATION OF ALVEOLAR DEFECTS – ANIMAL MODELS

Ana Rita Simões*, José Miguel Campos*, Ana Rita Caseiro*, Luís Miguel Atayde, José Domingos Santos, Ana Colette Maurício

Centro de Estudos de Ciência Animal (CECA); Instituto de Ciências, Tecnologias e Agroambiente (ICETA) da Universidade do Porto, Portugal



Introduction

The use of bone grafts is a reality in dental medicine in what concerns the correction of periodontal defects, prosthetic rehabilitation and the filling of large bone defects. Autologous bone graft remains the gold standard grafting substrate available in bone fusions, for small gaps and in critical defects (>5mm) as they tick most of the boxes for the ideal graft. However the complications associated with the second surgery for harvesting bone are not rare and have to do with donor-site morbidity and the limited amount of bone. Though xenografts are also widely used in oral medicine, they bring controversial considerations in what concerns the amount of organic material they may contain and the transmission of diseases. On the other hand, the absence of organic material doesn't allow the osteoinduction to occur.

Bonelike® (BL®) is an alternative synthetic bone graft, a glass-reinforced hydroxyapatite (HA) composite with osteoinductive and osteoconductive proprieties consisting in the incorporation of P_2O_5-CaO glass-based system within a HA matrix of spherical pellets with 250-500µm of diameter (Figure1&2). These proprieties of BL® have been confirmed in experimental models of bone regeneration in sheep and recently its application in orthopedic and oral pathologies is being tested in small animal clinics.

Aims and Objectives

The present study aimed to test the behaviour of the Bonelike® in alveolar defects and in critical ones, in animal models. These clinical cases intend to contribute to the development of new strategies, in the context of bone regeneration surgery, targeting a rapid restoration of the skeletal integrity and reduction of the associated morbidity where biomaterials such as ceramics play an important role. In order to promote regeneration, a cellular system has also been developing – mesenchymal stem cells isolated from the dental pulp (DPSCs) that when associated with the bone substitutes, decreases the time of regeneration and allows the formation of better quality bone from the histological point of view.

Materials and methods

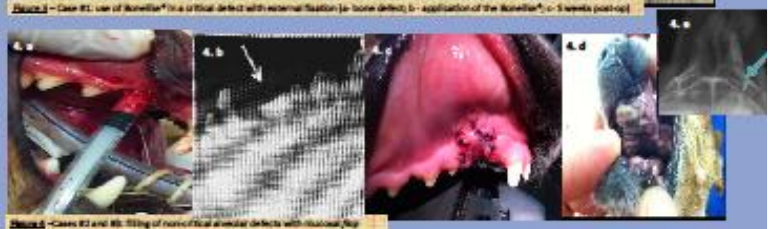
Case #1 mandibular fracture in a four year old dog, reduced externally, and in which the bone consolidation was confirmed six weeks after;

Cases #2, #3 and #4 alveolar filling of a defect created after dental extraction of the maxillar teeth with synthetic bone graft, in a five year old dog, a twelve year old dog and a six year old cat, respectively; in these cases, a mucosal *flap* was performed to avoid oronasal communication.

Radiologic evaluation (six weeks) and clinical examination were performed in different follow-up times in order to assess the efficacy of the surgical treatments.

The DPSCs were isolated from the dental pulp and characterized by karyotype, secretoma, immunocytochemistry, RT-PCR, flow cytometry and differentiation capacity in at least three distinct cell lines according to the *International Society of Cellular Therapy*.







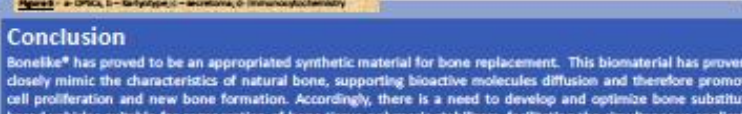


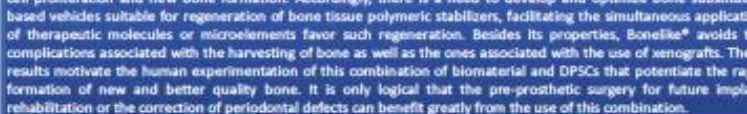
Results and Discussion

When compared with the surgical method for obtaining autologous material, the synthetic options have several advantages such as biocompatibility, osteoconduction, injectability, moldability, easy manipulation, minimally invasive procedure, less scar tissue (only one surgery needed) as well as the decreased risk of infection. Another advantage is its availability as it can be produced in large scale. The increasing demand for bone reconstruction materials has been motivating the research for new biomaterials.

The material that we tested - Bonelike®- showed satisfactory results in which concerns its osteoconductive and osteoinductive properties, as it promoted a quicker bone fusion without local or systemic reactions. The BL® granular forms (250-500 µm) facilitate the flow of biomaterial being less prone to clumping in the syringe and promote the best adaptation of the biomaterial to the anatomy of the defect. This characteristics increase its osteoconductive function mainly in the non-critical defects.

After *in vitro* characterization, the DPSCs were classified as mesenchymal stem cells according to the ISCT. Therefore they guarantee the presence of organic material and potentiate the osteoinduction.





Conclusion

Bonelike® has proved to be an appropriated synthetic material for bone replacement. This biomaterial has proven to closely mimic the characteristics of natural bone, supporting bioactive molecules diffusion and therefore promoting cell proliferation and new bone formation. Accordingly, there is a need to develop and optimize bone substitutes, based vehicles suitable for regeneration of bone tissue polymeric stabilizers, facilitating the simultaneous application of therapeutic molecules or microelements favor such regeneration. Besides its properties, Bonelike® avoids the complications associated with the harvesting of bone as well as the ones associated with the use of xenografts. These results motivate the human experimentation of this combination of biomaterial and DPSCs that potentiate the rapid formation of new and better quality bone. It is only logical that the pre-prosthetic surgery for future implant rehabilitation or the correction of periodontal defects can benefit greatly from the use of this combination.

This research was supported by Programa Operacional Regional do Norte (ON.2 – O Novo Norte), QREN, FEDER with the project "Bone Therapies: Terapias inovadoras para a regeneração óssea", and by Programa Operacional Competitividade e Internacionalização (P2020), Fundos Europeus Estruturais e de Investimento (FEEI) and FCT with the project "BioMate – A novel bio-manufacturing system to produce bioactive scaffolds for tissue engineering" with reference PTDC/EMS-SIS/7032/2014 and by COMPETE 2020, from ANI – Projectos IDT Empresas em Copromoção, Programas Operacionais POCI, by the project "Inoffo.Biomas - Relevent biomanufacturing systems by using an usability approach for *in situ* clinic temporary implants fabrication" with the reference POCI-01-0247-FEDER-017771.

COMPETE 2020 PORTUGAL 2020

References:

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Sousa RC, Lobato JV, Maurício AC, Hussain NS, Botelho CM, Lopes MA, Santos JD (2008). A clinical report of bone regeneration in maxillofacial surgery using bonelike synthetic bone graft. J Biomater Appl. 2008 Jan;22(4):373-85.

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Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.

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3th–4th December 2016 – **Poster Presentation** at Winter Meeting Portuguese Academy of Oral Medicine APMO, Lisbon, Portugal.

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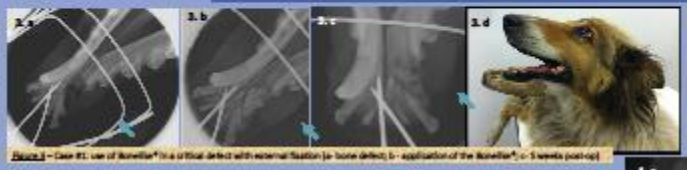
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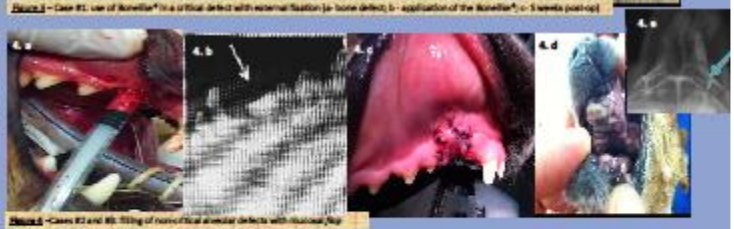
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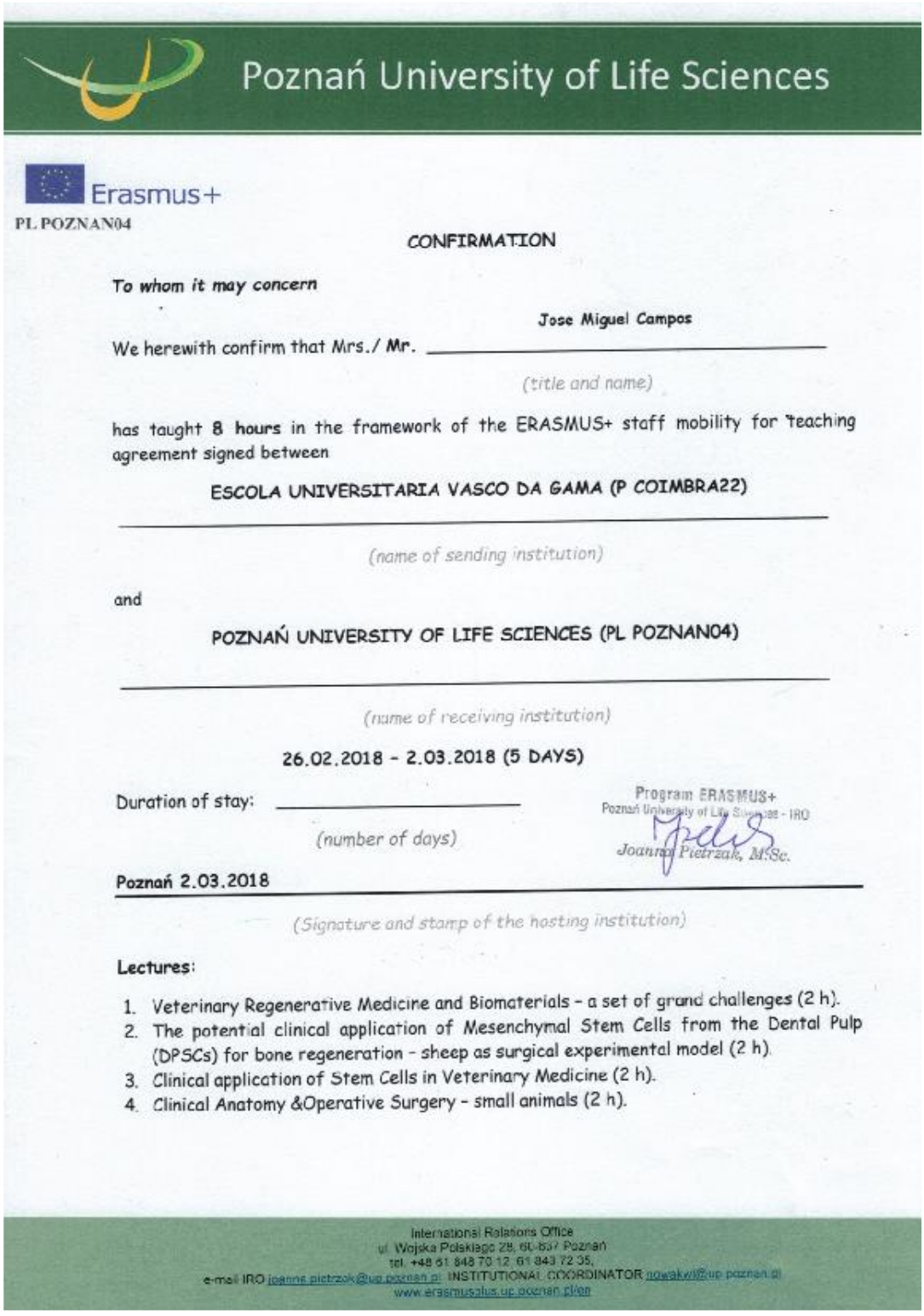
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26th February to 2nd March 2018, **Oral Communication** at Poznán University of Life Scienc, Poznán, Poland.



Poznań University of Life Sciences

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CONFIRMATION

To whom it may concern

We herewith confirm that Mrs./ Mr. Jose Miguel Campos

(title and name)

has taught **8 hours** in the framework of the ERASMUS+ staff mobility for teaching agreement signed between

ESCOLA UNIVERSITARIA VASCO DA GAMA (P COIMBRA22)

(name of sending institution)

and

POZNAŃ UNIVERSITY OF LIFE SCIENCES (PL POZNAN04)

(name of receiving institution)

26.02.2018 - 2.03.2018 (5 DAYS)

Duration of stay: _____

(number of days)

Program ERASMUS+
Poznań University of Life Sciences - IRO
Joanna Pietrzak, M.Sc.

Poznań 2.03.2018

(Signature and stamp of the hosting institution)

Lectures:

1. Veterinary Regenerative Medicine and Biomaterials – a set of grand challenges (2 h).
2. The potential clinical application of Mesenchymal Stem Cells from the Dental Pulp (DPSCs) for bone regeneration – sheep as surgical experimental model (2 h).
3. Clinical application of Stem Cells in Veterinary Medicine (2 h).
4. Clinical Anatomy &Operative Surgery – small animals (2 h).

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24th January 2018, **Oral Communication** at University of Cambridge, England, United Kingdom.



UNIVERSITY OF CAMBRIDGE
Department of Veterinary Medicine

CERTIFICATE OF PARTICIPATION

This is to certify that

José Miguel Campos

Presented a Seminar at the Department of Veterinary Medicine
Departmental Seminar Series on 24th January 2018

UNIVERSITY OF CAMBRIDGE
Department of Veterinary Medicine
Madingley Road
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Fiona Roby
Research and Graduate Education Administrator
Department of Veterinary Medicine
University of Cambridge

02/02/2018
Date